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THE FLOW OF BLOOD IN NARROW TUBES

II. THE AXIAL STREAM AND ITS FORMATION, AS DETERMINED BY CHANGES IN OPTICAL DENSITY

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(Accepted for publication 3rd May, 1954.)

It has been abundantly confirmed that suspensions of particles show non-Newtonian flow, particularly in narrow tubes, and many attempts have been made to establish exact or empirical laws for their behaviour in viscometers (Eirich and Riseman, 1949). The whole subject, as it applies to blood, has been extensively reviewed by Bayliss (1952); references to much early work are contained in a paper by Rothmann (1914). Several factors are concerned in the explanation of this phenomenon, and the experiments reported in this paper provide evidence that at least two are of importance; namely, the formation of a "marginal zone" of fluid, free of particles, surrounding an "axial stream" of cells; and changes in the degree of orientation of the cells.

These points were made by Hess (1911) who, in criticizing the approach adopted by Hatschek (1910), wrote that "z. B. wir haben nicht in Berechnung gezogen den Einfluss der Art der Oberfläche der Blutkörperchen, ihre Rotation, ihre ungleiche Verteilung in Gefässquerschnitt." Hatschek did, however, state that spherical particles in such a situation might be distorted to ellipsoids if they were not rigid; Vand (1948) made the same point. It is possible that red cells may be distorted while flowing, but the only evidence of the importance of their elasticity appears to be that of Berczeller and Wastl (1924a, b, 1926). They found that changes in the viscosity of blood, due to ageing, were accounted for by the changes in the red cells themselves. They measured these changes by determining the viscosity of samples of cells packed by centrifugation.

Haller (1932), Kuhn (1932) and Kuhn and Kuhn (1945), have discussed the influence of shearing on the viscosity of suspensions of deformable particles (e.g., coiled polymers), while Müller (1948a) and Züger (1948) have shown, with model experiments, the distortion of liquid globules flowing in a tube. While the shape of a red cell under shear in a capillary tube is not known, the ready deformability seen in living preparations, e.g., the rabbit ear chamber, makes it quite possible that distortion during flow does actually occur. Such distortion could lead to alterations in the optical properties of the suspension.

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[1]Research Fellow, Department of Medicine.

In connection with this, the finding of Orskov (1934, 1935) may be important. He found that, with increase in cell-volume, a suspension of red cells transmitted more light. Techniques based on this phenomenon have since been successfully applied to the investigation of the osmotic behaviour of red cells and red cell ghosts (Teorell, 1952; Hunter, 1949a, b, 1953).

The presence of an axial stream of cells has long been noted by many workers on the circulation in small vessels in living tissues, and such phenomena as "plasma skimming" are satisfactorily explained by the presence of the accompanying "marginal zone." The effect of this type of flow on the apparent viscosity of blood in small vessels has recently been discussed by Isenberg (1953). The cell-free layer at the boundary of the tube permits the development there of high rates of shear, so that the fluid behaves as though it "slips" at the wall; the greater the slip the less the apparent viscosity. Isenberg obtained an equation relating the "asymptotic" value of the apparent viscosity of a sample of blood to the diameter of the capillary tube in which it was measured. The equation agreed well with the results of Fahraeus and Lindqvist (1931). The observation that in narrow tubes the apparent viscosity of a sample of blood decreases with increasing rate of shear, finally approaching its "asymptotic" value where it is uninfluenced by further increase in shear, may in part be explained by the corresponding increase in the cell-free marginal zone. The present experiments indicated that this occurred.

Papers by Drabkin and Singer (1939) and Kramer, *et al.* (1951), contain reference to the observation that blood reflects and transmits more light when flowing than when stationary. This has been ascribed to the orientation of the red cells. A similar observation has been made in this laboratory during other experiments; in the pulsatile flow of blood in the vessels of the preformed tissue of the rabbit ear chamber, as the blood moved forward the centre of the vessel grew bright, darkening again as the flow slowed. Jäger (1935) has reported the same appearance in the small vessels of the frog.

The apparent viscosity of suspensions of ellipsoids has been found to depend upon the shear at which the measurement is made (see Alexander and Johnson, 1949). With higher shear the orientation of the particles is increased, and the dissipation of energy by their rotation decreased, so that the measured viscosity falls. For colloidal systems under Brownian agitation, the position of statistical maximum orientation relative to the lines of shear depends upon the rotational diffusion constant. For larger particles, where Brownian movement is negligible, the maximum orientation is in the line of shear, but the particles are constantly in rotation, as Kuhn has stated, "ein in der strömenden Flüssigkeit schwebendes Teilchen hinsichtlich der Stellung seiner Längsäche nie zur Ruhe kommt, sondern sich dauernd im selber Sinne (der durch das Strömungsgefälle vorgeschrieben ist) um dreht." An interesting paper by Mason (1950) dealt with the behaviour of paper fibres, and Eirich, Bunzl and Margaretha (1936) have described the

passage of suspensions of small glass rods through capillaries. They described the formation of a particle-free marginal zone. Trevelyan and Mason (1951) made careful observations of the rotation of suspended particles in a Couette viscometer, and found that the theoretical predictions were fulfilled. Trevan (1918) considered that account should be taken of the energies of rotation of red cells in flowing blood, particularly when the cell concentration was less than 0·45 by volume. Below this concentration the cells, being more spaced out, could rotate more freely. The experiments of Brundage (1935) showed that suspensions of red cells in a rotating viscometer reached their "asymptotic" apparent viscosity at rates of shear much lower than those required in a capillary tube. That is, the maximum orientation of the cells was more easily achieved in fluid where the shear was uniform from point to point. In fact, in his small apparatus the shear would not have been quite uniform across the annulus, but was far more nearly so than would be found in a capillary tube, where it varies from zero at the axis to a maximum at the wall.

In order to investigate the orientation effects and the marginal zone formation, with increasing rate of flow, experiments were conducted with suspensions of cells driven through fine glass capillaries in the apparatus previously described (Taylor and Robertson, 1954). Some theoretical consideration has also been given to the forces involved in the formation of the axial stream.

Materials and Methods.

Citrated blood was used, the cells being suspended in isotonic saline after having been separated from the plasma by centrifugation and washing twice. For convenience, in the numerous pilot experiments, unwanted blood was obtained from the Red Cross Transfusion Service. In order to determine the effect of storage on the phenomena, samples of blood, similarly citrated, were examined when fresh and after 1, 3, 6 and 8 days' storage in the refrigerator (at approximately 40° F.). In those experiments where the haematocrit value was varied, saline was used as the diluent.

Technique.

Before use the reservoir was cleaned with strong chromic acid and then rinsed repeatedly with distilled water followed by isotonic saline. The capillary tube was cleaned by drawing these fluids through it by suction. The suspension was placed in the reservoir, which was then sealed, and the stirrer set in motion. A positive pressure of 10 mm. Hg. was applied to the fluid by the compressed air system. Readings were taken of the light intensity at a succession of points across the tube, beginning at the wall and progressing to three of four positions past the centre of the tube. At each point the intensity was measured as the suspension was flowing under pressure heads of 10, 20, 40, 80, 160, 240 and 300 mm. Hg. The base pressure of 10 mm. Hg. was found to be necessary as otherwise, at the edge of the tube, the passage of individual cells caused fluctuations in the light intensity which made it impossible to obtain accurate levels for comparison. The slow passage of an occasional leucocyte adhering to the wall also sometimes interfered with the readings.

Changes in the optical density at each point at the different pressures were calculated by subtracting the logarithm (base 10) of the light intensity at 10 mm. Hg. from the

logarithm of that at the given pressure. A positive value of this difference indicated an increase in the light transmission and *vice versa*.

The tube used in these experiments had a diameter of 0·190 mm., and a length of 14·4 cm.

RESULTS.

Variations of haematocrit value.

The findings are shown in Figs. 1–5, the haematocrit values being respectively 57, 38, 33, 24 and 17 p.c. The ordinates are decreases in optical density from that at a pressure of 10 mm. Hg. In order not to crowd the graphs, the values are given only for three pressures, 20 mm. Hg. (solid circles), 80 mm. Hg. (open circles) and 240 mm. Hg. (crosses). The readings just inside the edge of the tube are at station 1 on the abscissa. The centre of the tube lies at about the thirtieth station. Table 1 gives, as an example, the readings obtained with a 24 p.c. suspension.

TABLE 1

Station	L 10	L 20	D 20	L 80	D 80	L 240	D 240
1	68·0	68·0	0·000	68·5	0·003	75·0	0·042
2	68·0	68·0	0·000	69·0	0·006	74·0	0·037
3	81·0	82·0	0·005	89·0	0·041	100·0	0·091
4	60·0	62·0	0·014	65·5	0·038	77·0	0·108
5	58·0	57·0	−0·008	57·0	−0·008	65·0	0·049
6	52·0	51·0	−0·008	51·0	−0·008	58·5	0·051
7	47·5	46·0	−0·014	45·0	−0·023	50·0	0·022
8	59·0	57·0	−0·015	56·0	−0·023	61·5	0·018
9	52·0	50·5	−0·013	49·0	−0·026	54·0	0·016
10	46·5	45·5	−0·009	44·5	−0·019	48·0	0·014
11	42·0	41·0	−0·010	40·0	−0·021	43·0	0·010
12	39·0	37·0	−0·023	36·5	−0·029	38·5	−0·006
13	35·0	34·5	−0·006	34·0	−0·013	36·0	0·012
14	33·5	33·0	−0·007	32·5	−0·013	34·5	0·013
15	29·0	28·0	−0·015	28·0	−0·015	29·5	0·007
16	25·0	25·0	0·000	24·5	−0·009	26·5	0·025
18	20·0	20·5	0·011	20·5	0·011	22·0	0·041
20	17·5	17·5	0·000	18·0	0·012	19·5	0·047
22	15·5	15·5	0·000	16·5	0·027	18·0	0·065
24	32·0	32·5	0·007	35·0	0·039	39·0	0·086
26	30·5	31·5	0·014	34·5	0·053	38·5	0·101
28	29·5	31·0	0·021	34·5	0·068	38·5	0·116
30	29·5	31·5	0·028	34·0	0·061	38·5	0·116
32	31·0	32·5	0·020	35·0	0·053	39·0	0·111

L, Light intensity, galvanometer reading.
D, optical density change relative to L 10.
The numbers in suffix refer to the pressure in mm. Hg.

It was found in all cases, with increasing rate of flow, that there was an increase in the light transmission at the edge of the tube. As the point of observation was moved towards the centre of the tube, the magnitude of the change depended upon the haematocrit value. The general form of the graphs

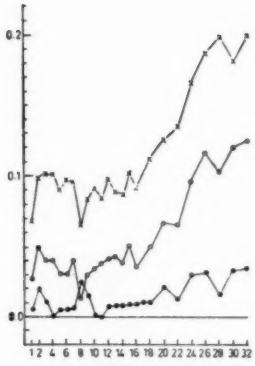

Fig. 1.

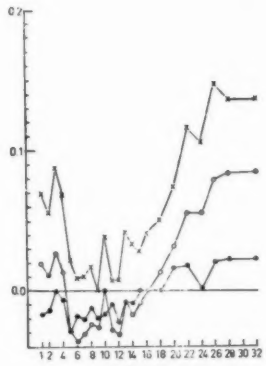

Fig. 2.

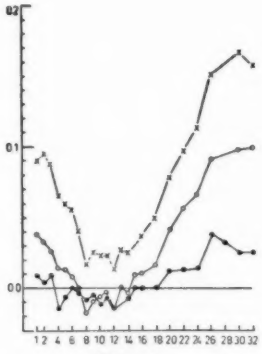

Fig. 3.

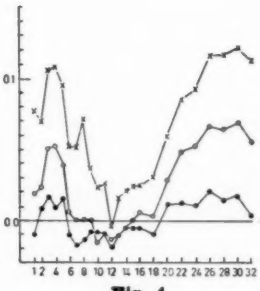

Fig. 4.

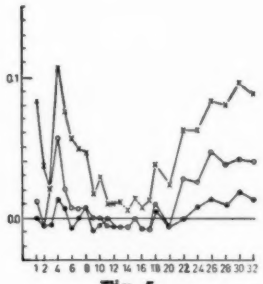

Fig. 5.

Figs. 1–5 show changes in optical density of different suspensions of red cells in saline when the pressure head was varied. Details in text.

could be distinguished as consisting of two main features, a marginal and a central peak. In the graph for the behaviour of the 57 p.c. suspension, the emergence of the central peak was so far towards the wall of the tube that the trough between the two peaks in the other graphs did not appear. It was further observed that while the height of the central peak was less in the specimens of lower haematocrit value, and its emergence nearer to the wall, the height of the marginal peak was approximately the same in all the graphs.

The effect of storage.

The form of the graphs obtained with specimens of varying age was similar to that shown in Figs. 1–5, except for that obtained with the specimen which had been stored for one day. In this the marginal peak was present as usual, but the central rise occurred only in the 240 mm. Hg. line, all readings from about 0·8 of the radius inward being negative. A second specimen, stored under the same conditions, gave the usual response. This one anomalous finding was of considerable interest, and is mentioned because specimens in which blood was used, which had been collected in other anti-coagulants, also gave a similar graph. Preliminary investigations suggest that the pH of the suspension is the determining factor.

DISCUSSION.

The interpretation of the findings was based on the assumption that three principal changes were involved; namely, the clearance of cells from the edge of the tube, a packing in the axial stream, and an increase in the degrees of orientation of the cells in the axial stream. These changes were considered to be responsible for, respectively, the marginal peak, the trough, and the central peak of the graphs. The changes were, of course, inter-related, but it is simplest to consider them separately.

To determine the effect on light transmission of changes in the cell population, we take the case in which cells removed from the marginal annulus are

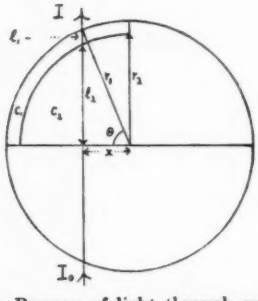

Fig. 6. Passage of light through suspension. Details in text.

redistributed in the axial stream. While this is not quite the case in the flowing blood, it is the most useful model to consider.

In Fig. 6, the beam of light passes through a point on the diameter, distant x from the centre of the tube. The illumination is considered to be parallel, and refraction is neglected. The error in making these assumptions is relatively small, as was indicated in a previous paper (Taylor and Robertson, 1954).

If the initial concentration C_0 of the absorbing medium is uniform over the whole tube, the optical density is given by the Lambert-Beer formula.

$$D_2 = \log I - \log I_0 = 2k(l_1+l_2)\,C_0 \qquad \text{(1)}$$

where k = extinction coefficient of the medium

I_0 = intensity of the incident beam

I = intensity of the emergent beam

l_1 = length of the light path through the marginal annulus.

l_2 = length of the light path through the axial region.

Consider now that the concentration in the marginal annulus (inner radius r_2) is reduced to C_1, and the removed material redistributed in the inner region; the concentration here will increase to

$$C_2 = C_0+(C_0-C_1)\left\{\left(\frac{r_1}{r_2}\right)^2-1\right\} \qquad \text{(2)}$$

So that, where $r_1 \geqslant x \geqslant r_2$, the optical density difference from its original value is given by

$$\begin{aligned} D_1' &= 2l_1k(C_0-C_1) \\ &= 2k(C_0-C_1)r_1 \sin\theta \end{aligned} \qquad \text{(3)}$$

and where $r_2 \geqslant x \geqslant 0$, from (2) we obtain

$$D_2' = 2k\left[l_1(C_0-C_1)-l_2(C_0-C_1)\left\{\left(\frac{r_1}{r_2}\right)^2-1\right\}\right] \qquad \text{(4)}$$

which, putting

$$2k(C_0-C_1) = a$$

$$x = r_1 \cos\theta$$

$$\frac{r_1}{r_2} = \beta$$

we may write in the form

$$D_2' = ar_1(\sin\theta-\beta.\sqrt{1-\beta^2\cos^2\theta}) \qquad \text{(5)}$$

This function has been computed for a value of $\beta=1{\cdot}1$, and expressed in terms of ar_1, and is graphed in Fig. 7. It has a maximum value at $x=r_2$, of $0{\cdot}415$, and a minimum at $x=0$, of $-0{\cdot}210$.

Consideration of this figure makes it clear that a decrease in the cell concentration at the edge of the tube will have a maximum effect on the light transmission near the margin, where it will be increased. As the point of observation is brought nearer to the centre of the tube, the effect of the increased

cell concentration becomes more and more marked, outweighing the effect of clearance from the marginal zone, so that in the central regions the light transmission decreases.

It was assumed, for purposes of the above discussion, that the cells leaving the marginal zone were all redistributed in the axial stream; in fact, however, although the effect of formation of a marginal zone will be to raise the concentration of cells in the axial stream, the condition that must be satisfied is not that the number of cells in a cross-section of the tube must remain constant, but that, after equilibrium, the number of cells entering and leaving the tube in a given time must be constant. This condition is expressed by the equation

$$\int_0^a 2\pi.a_0.v.r\,dr = \int_0^{\gamma a} 2\pi.a_r.v.r.dr \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (6)$$

where a = radius of the tube
r = distance from axis of tube
v = velocity at r
a_1 = concentration of cells in the reservoir, assumed constant across the entrance of the tube
a_r = concentration of cells at r
γ = ratio of radius of axial stream to that of tube

Now, the total quantity of fluid passing per unit time is given by

$$Q_t = 2\pi\int_0^a r.v.dr = 2\pi\int_{\gamma a}^a r.v.dr + 2\pi\int_0^{\gamma a} r.v.dr = Q_p + Q_c$$

so that the volume discharged by the axial stream is given by

$Q_t - Q_p = Q_c$, where Q_p is that discharged by the marginal annulus. Thus, if we take α as the average value of a_r over the axial stream, we may write

$$\alpha = a_0 \frac{Q_t}{Q_t - Q_p} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (7)$$

But, by the Poiseuille formula, the outflow is given by

$$Q_t = \frac{\Delta P.\pi a^4}{8\eta l}$$

where ΔP = pressure drop over the tube
l = tube length
η = apparent viscosity coefficient at a given pressure.

The outflow from the marginal annulus is given by

$$Q_p = 2\pi\int_{\gamma a}^a \frac{\Delta P}{4\mu l}(a^2 - r^2).r.dr = \frac{\Delta P.\pi}{2\mu l}\left[\frac{a^2 r^2}{2} - \frac{r^4}{4}\right]_{\gamma a}^a$$

where μ is the coefficient of viscosity of the suspending medium.
This reduces to

$$\frac{\Delta P \pi a^4}{8\mu l}(1-\gamma^2)^2$$

so that, substituting in (7) and simplifying, we obtain, putting $\phi = \eta/\mu$, i.e. the apparent viscosity of the suspension relative to that of the suspending medium,

$$\alpha = a_0 \frac{1}{1-(1-\gamma^2)^2\phi} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (8)$$

This indicates that the average concentration over the axial stream will be higher than that in the reservoir; and thus, while in practice the changes in optical density with formation of the axial stream will differ from those of the model considered, they will, in general, resemble those shown in Fig. 7. That is, with increase in rate of flow and width of marginal zone, the light transmission will increase near the edge of the tube, pass through a maximum, and then decrease towards the centre of the tube.

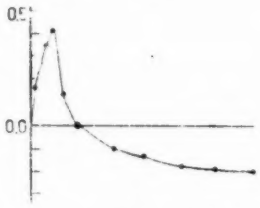

Fig. 7. Optical density change due to marginal clearance. Wall of tube at left, centre at right. Ordinated: magnitude of change in terms of $\alpha\gamma_1$ (see text).

Vejlens (1938) and Bayliss (1952) have both discussed the mechanism of formation of the axial stream, but have not reached any definite conclusions. The main difficulty of the problem is to obtain and balance two forces, one tending to disperse the particles, and the other moving them inward from the wall of the tube. The following consideration is also incomplete, but pursues the argument originally proposed by Vejlens.

The particles in a suspension flowing through a tube will be set in rotation by the shear, and their presence will modify the distribution of velocities so that the suspension will not flow with a parabolic velocity profile. This was well demonstrated by Vejlens (1938), who made an ingenious apparatus modelled on that of Reynolds (1883), in which dye was introduced through a micro-pipette into blood flowing in a capillary. The "front" of the advancing dye in water was the usual parabola, but in blood was greatly blunted, and as Vejlens wrote, was "approximately the same as in a turbulently flowing liquid." Müller (1948b) came to much the same conclusion, and stated that "in der Regel führt ein Teil der Zusatzteilchen komplizierte translatorische und rotatorische Bewegungen aus. Obwohl nun die Suspensionsflüssigkeit notwendigerweise auch Rotationen ausführen muss, ist diese Art der Strömung doch von der turbulenten Strömung streng abzusondern." A recent brief communication by Bloch (1953) reported that high-speed cinematography (at 3,000 frames/sec.) of flowing blood disclosed "turbulent flow with continuous reorientation of cellular components." There does not appear at present to be any term apt to describe this type of flow, which is, by definition, neither turbulent nor laminar; it is suggested that

"mixed" may be a suitable adjective. The presence of mixed flow must have some effect upon the diffusion of substances from the stream. Sangren and Sheppard (1953), in a theoretical treatment of the exchange of a labelled substance between liquid flowing in a vessel and an external compartment, considered their assumption of uniform mixing of the contents of the vessel to be unrealistic. However, it was probably a nearer approach to reality than an assumption of laminar flow.

The dispersion and disorientation of the particles in the axial stream must also be furthered by their collisions. For discussions of the nature of particle collisions in sheared suspensions reference should be made to the papers of Smoluchowski (1918), Goodeve (1939, 1948), Vand (1948), Mason (1950), Mooney (footnote, 1951); and to the particularly illuminating paper by Manley and Mason (1952), in which were described their direct observations of colliding spheres in a contra-rotating Couette apparatus.

Other forces which would tend to disperse the particles, e.g., Brownian agitation and electrical changes, were too small in the system studied to have any significant effect: the first because of the relatively large dimensions of the red cell, and the second because of the high ionic concentration of the medium (Abramson, Moyer and Gorin, 1942; Alexander and Johnson, 1949).

Whereas, then, in the axial stream a particle will be subjected to fluctuating forces inward and outward, due to collisions and the disturbed nature of the "mixed" flow, in the marginal zone, where the flow remains laminar, the hydrodynamic forces will be steady. One may arrive at some notion of the inward-acting force on a particle in the marginal zone by following Vejlens in making a dimensional analysis. He took, as the terms involved, the radius of the particle, the viscosity of the medium, the velocity of flow at the point considered, and the least distance between the particle and the wall of the tube. It appears that particles in a uniformly sheared medium, although they rotate, do not move across the lines of shear. Jeffery (1923) suggested that particles in suspension would move in such a way as to minimize the dissipation of energy, and in flow through a tube would thus tend to collect about the axis in the region of lowest shear. Even in the Couette apparatus, where the non-uniformity of shear is slight compared to that in a capillary tube, Trevelyan and Mason (1951) did detect slight translation of particles into regions of lower shear. It is, therefore, reasonable to regard non-uniformity of shear as important in the present problem, and to include it in the analysis. It is expressed as the space rate of change of the rate of shear.

For the force (F) acting on a particle in the marginal zone, at a distance (R) from the axis of the tube, we may write

$$F = k.a^{\alpha}.\mu^{\beta}.\left(\frac{dv}{dr}\right)^{\gamma}.\left(\frac{d^2v}{dr^2}\right)^{\lambda} \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (9)$$

where the following terms are included:

a, the diameter of the particle

μ, the coefficient of viscosity of the suspending medium

$\frac{dv}{dr}$, the rate of shear at R.

$\frac{d^2v}{dr^2}$, the space rate of change of the shear at R

R, the distance of the particle from the axis of the tube

α, β, γ, λ, k, constants.

The dimensional equation is

$$M.L.T^{-1} = L^{\alpha}.M^{\beta}L^{-\beta}.T^{-\gamma}.\ {}^{-\lambda}T^{-\lambda}$$

whence $\lambda = (1-\gamma)$, $\alpha = (3-\gamma)$, so that (9) becomes

$$F = ka^3\mu\left(\frac{\frac{dv}{dr}}{a\frac{d^2v}{dr^2}}\right)^{\gamma}\frac{d^2v}{dr^2} \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (10)$$

For laminar flow in a capillary tube we have

$$\frac{dv}{dr} = \frac{-\Delta P}{2\mu l}R$$

which, on differentiation yields

$$\frac{d^2v}{dr^2} = \frac{-\Delta P}{2\mu l}$$

and substituting these values in (10), we obtain the relationship

$$F = -ka^3\left(\frac{R}{a}\right)^{\gamma}\frac{\Delta P}{2l} \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (11)$$

This approach neglects the disturbance of the velocity field due to the insertion of the particle, and hence is to be regarded only as an approximation. For the general case given by (10), the value of γ must be such that the force is not definite when $dv/dr=0$, or when $d^2v/dr^2=0$; that is $1\geq\gamma\geq0$. Theory does not allow any closer determination of the effect of variation of the position of the particle in relation to the wall of the tube, on its inward transport. It can be said, however, that the maximum inward transport will be at the wall of the tube, falling off in an undetermined way as the axis is approached. It is emphasized, on the other hand, that once the particle enters the region of mixed flow this force no longer operates; in the axial stream the conditions under which (11) was established no longer exist. It is further emphasized that these phenomena are significant only in small vessels and narrow tubes, where the dimensions of the particles are comparable to the diameter of the tube in which they flow. In general, the flow of blood in larger tubes resembles that of a homogeneous fluid (Hahn, Donald and Grier, 1945; Ralston and Taylor, 1945; Ralston, Taylor and Elliot, 1947). Turbulence in the accepted sense was

thought by Coulter and Pappenheimer (1949) to be confined to a narrow peripheral plasma sleeve; Garb (1944), McDonald (1952a, b) and Rossi, Powers and Dwork (1953), however, considered that it could involve the whole stream.

The orientation of cells in the axial stream of flowing blood will be influenced not only by the shearing (as was discussed in the introduction) but by the concentration of the particles present, alignment of one tending to bring about alignment of the others. The degree to which this mechanism is effective will increase with increasing concentration. Further, the greater the degree of orientation of the system, the fewer will be the collisions between the particles, and the less the tendency for the axial stream to disperse. Thus, with increasing pressure head, if the reasoning underlying (11) is correct, the inward drift of particles will be enhanced to a greater extent than the outward, and equilibrium will be established between the two regions nearer the axis of the tube. That is, the diameter of the axial stream will decrease.

The orientation of the particles also influences the transmission of light by the system. It is considered that the "central peak" obtained in the present experiments represented the effect of increasing orientation of the cells. It has not been possible to set up any satisfactory mathematical description of the changes in optical properties which would be anticipated from an increasing orientation. It was assumed, however, that an orientated system of particles would scatter less and so transmit more light than a random one. Experimental support for this view has been obtained by observing the flare about the image of the illuminated slit focused across the diameter of the capillary tube. When the rate of flow was low (10 mm. Hg. pressure), a curved area of the cross-section beyond the edges of the image was seen to be illuminated by light scattered on its passage through the suspension. With increasing rate of flow this area contracted, and the edges of the image became sharper, denoting a decrease in the degree of scattering. The increased orientation was thus taken to have decreased the extinction coefficient of the medium. The change detected in the optical density at any given point was therefore proportional to the length of the light path through the medium at that point. That is, the orientation effect was maximum at the centre of the tube, decreasing towards the edge.

It is now possible to combine the various matters discussed above and summarize the results.

In the marginal zone, where laminar flow was preserved for some small distance inwards, the particles were acted upon by an inward-directed force, and thus impelled into the axial stream. With increasing pressure applied, this inward drift was increased, the loss of particles leading to a reduction in the optical density of the edges of the stream. This effect appeared in the graphs as the marginal peak.

In order to satisfy the condition that the number of particles entering and leaving the tube should be constant (6), the concentration in the axial stream

increased, giving rise to optical density changes, with increasing pressure, of the kind shown in Fig. 7. This, and the foregoing change, accounted for the marginal peak and the trough. In the specimen of high cell concentration (Fig. 1), the trough did not appear, being masked by the large orientation effect.

In the axial stream, collisions and rotations of the particles tended to disperse them outward, a condition of equilibrium eventually being reached when the packing tendency of the marginal zone was just balanced. The progressive shift inwards of the emergence of the central peak, with decreasing cell concentration, was probably due not only to the smaller diameter of the axial stream in those specimens, but to the presence of a layer of cells in which orientation was already considerable at the base pressure. Any increase of pressure, then, while it increased the packing (and hence increased the optical density) did not decrease the optical density to any extent as a result of increasing orientation. In the central regions of the axial stream, however, the orientation being originally less, could be increased, and when the point of observation included light passing through these regions, orientation effects began to be detected with increase of pressure. Such a state of affairs would explain the widening of the trough and the decrease in height of the central peak found with the lower haematocrit values. A further factor probably responsible for the decrease in the "orientation effect" at lower cell concentrations was the lessening tendency of the system to be organized by those particles which were orientated. It must also be remembered that with lower cell concentrations the extent to which the suspension could be "packed" was greater than that at high concentrations, giving larger negative values of the optical density change near the centre of the tube, thus reducing the central peak and widening the trough.

SUMMARY.

A discussion of the nature of flow in suspensions of red cells in narrow capillaries, and an interpretation of the changes in light transmission through a series of points across streams flowing under different pressures and containing different concentrations of cells has suggested that a marginal zone was present in which, with increasing rate of flow the cell concentration was progressively reduced. This accords with the theories proposed to explain the anomalous viscosity of blood in terms of the slippage at the wall.

The concentration of cells in the axial stream underwent a compensatory increase. This does not conflict with the observations of Fahraeus (1929) that with increasing rate of flow the concentration of cells *in the tube* was decreased.

With increasing rate of flow the particles in the axial stream showed increasing orientation.

Theoretical considerations, and the observation of Bloch (1953), support

the contention of Vejlens (1938) and Müller (1948) that flow in the axial stream has some resemblance to turbulence. It is proposed that flow of this type be termed "mixed flow." The presence of mixed flow should be taken into account in any theory of the exchange of substances between blood and the extracapillary tissues.

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REFERENCES.

Abramson, H. A., Moyer, L. S. and Gorin, M. H. (1942): Electrophoresis of Proteins and the Chemistry of Cell Surfaces. Reinhold Publishing Corp., New York.
Alexander, A. E. and Johnson, P. (1949): Colloid Chemistry, Oxford.
Bayliss, L. E. (1952): Rheology of Blood and Lymph, in Deformation and Flow in Biological Systems, Ed. E. Frey-Wyssling, North Holland Publishing Co., Amsterdam.
Berczeller, L. and Wastl, H. (1924a): Pflügers Arch., 203, p. 436.
Berczeller, L. and Wastl, H. (1924b): Biochem. Z., 153, p. 110.
Berczeller, L. and Wastl, H. (1926): *Ibid.*, 167, p. 195.
Bloch, E. H. (1953): Anat. Rec., 115, p. 283.
Brundage, J. T. (1935): Amer. J. Physiol., 110, p. 659.
Coulter, N. A. and Pappenheimer, J. R. (1949): *Ibid.*, 159, p. 401.
Drabkin, D. L. and Singer, R. B. (1939): J. biol. Chem., 129, p. 739.
Eirich, F., Bunzl, M. and Margaretha, H. (1936): Koll. Zeitschr., 75, p. 20.
Eirich, F. and Riseman, J. (1949): J. polymer Sc., 4, p. 417.
Fahraeus, R. (1929): Physiol. Rev., 9, p. 241.
Fahraeus, R. and Lindqvist, T. (1931): Amer. J. Physiol., 96, p. 562.
Garb, S. (1944): Amer. Heart J., 28, p. 568.
Goodeve, C. (1939): Trans. Faraday Soc., 35, p. 342.
Goodeve, C. (1948): Proc. Internat. Congress Rheology, p. 2–5.
Hahn, P. F., Donald, W. D. and Grier, R. C. (1945): Amer. J. Physiol., 143, p. 105.
Haller, W. (1932): Koll. Zeitschr., 61, p. 26.
Hatschek, E. (1910): *Ibid.*, 7, p. 301.
Hess, W. (1911): Pflügers Arch., 140, p. 354.
Hunter, F. R. (1949a): Science, 109, p. 119.
Hunter, F. R. (1949b): J. cell. comp. Physiol., 33, p. 199.
Hunter, F. R. (1953): *Ibid.*, 41, p. 387.
Isenberg, I. (1953): Bull. math. Biophys., 15, p. 149.
Jäger, A. (1935): Pflügers Arch., 235, p. 705.
Jeffery, G. B. (1922): Proc. Roy. Soc. London (A.), 107, p. 161.
Kramer, K., *et al.* (1951): Amer. J. Physiol., 165, p. 229.
Kuhn, W. (1932): Z. phys. Chem. (Abt. A.), 161, p. 1.
Kuhn, W. and Kuhn, H. (1945): Helv. chim. Acta, 28, p. 1533.
McDonald, D. A. (1952a): J. Physiol., 118, p. 328.
McDonald, D. A. (1952b): *Ibid.*, 118, p. 340.
Manley, R. St.J. and Mason, S. G. (1952): J. colloid Sci., 7, p. 354.

Mason, S. G. (1950): Pulp and Paper Magazine of Canada, April.
Mooney, M. (1951): J. colloid Sci., 6, p. 162.
Müller, A. (1948a): Helv. Physiol. Acta, 6, p. 32C.
Müller, A. (1948b): *Ibid.*, 6, p. 181.
Ørskov, S. L. (1934): Biochem. Z., 269, p. 349.
Ørskov, S. L. (1935): Ibid., 279, p. 241.
Ralston, H. J. and Taylor, A. N. (1945): Amer. J. Physiol., 144, p. 706.
Ralston, H. J., Taylor, A. N. and Elliot, H. W. (1947): *Ibid.*, 150, p. 52.
Reynolds, O. (1883): Collected Papers, 2, p. 51. Cambridge University Press, 1901.
Rossi, H. H., Powers, S. H. and Dwork, B. (1953): Amer. J. Physiol., 173, p. 103.
Rothmann, M. (1914): Pflügers Arch., 115, p. 318.
Sangren, W. C. and Sheppard, C. W. (1953): Bull. math. Biophys., 15, p. 387.
Smoluchowski, M. V. (1918): Z. phys. Chem., 92, p. 129.
Taylor, M. (1953): Austral. J. exp. Biol., 31, p. 533.
Taylor, M. and Robertson, J. S. (1954): *Ibid.* (in press).
Teorell, T. (1952): J. gen. Physiol., 35, p. 669.
Trevan, J. W. (1918): Biochem. J., 12, p. 60.
Trevelyan, B. J. and Mason, S. G. (1951): J. coll. Sci., 6, p. 354.
Vand, V. (1948): J. phys. coll. Chem., 52, p. 277.
Vejlens, G. (1938): Acta Path. Microbiol. Scand., Supp. 33.
Züger, J. (1948): Diss. Fribourg. (cited by Müller, 1948).

THE USE OF PURE ENZYMIC SYSTEMS FOR THE INCORPORATION OF ^{32}P INTO ADENOSINE TRIPHOSPHATE AND PHOSPHOCREATINE

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In investigations involving the adenylic acid system it is sometimes desirable to use adenosine triphosphate (ATP) or adenosine diphosphate (ADP) labelled with ^{32}P in one or more positions. Lindberg and Ernster (1952) made use of ADP labelled in either the P atom attached to the ribose (α-P) or terminally (β-P); and Hems and Bartley (1953) prepared ATP labelled in both the middle (β-P) and terminal (γ-P) P atoms for the investigation of transphosphorylations involving inosine and adenosine polyphosphates.

The present communication describes methods for the preparation of ^{32}P-labelled ATP which differ from those mentioned above in that *in vitro* reactions catalysed by pure enzymic systems are employed. The methods offer means of introducing a label into any one or more of the P atoms in the ATP or ADP molecule, and also an enzymic means of preparing ^{32}P-labelled phosphocreatine (PC).

EXPERIMENTAL.

Principles of method.

The following reactions are involved:

$$ATP \xrightarrow{\text{myosin}} ADP + PO_4^{-3} \quad \text{I (a)}$$

$$ATP + \text{glucose} \xrightarrow{\text{hexokinase}} ADP + \text{glucose-6-P} \quad \text{I (b)}$$

$$ATP + AMP \text{ (adenylic acid)} \underset{}{\overset{\text{myokinase}}{\rightleftharpoons}} 2\ ADP \quad \text{II}$$

$$ATP \xrightarrow{\text{apyrase}} AMP + 2PO_4^{-3} \quad \text{III}$$

$$ADP + PC \underset{}{\overset{\text{creatine phosphokinase}}{\rightleftharpoons}} ATP + \text{Creatine} \quad \text{IV}$$

$$AMP + 2\ PC \xrightarrow{\text{creatine phosphokinase \& myokinase}} ATP + 2\ \text{Creatine} \quad \text{V}$$

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Reaction IV is reversible, but, as described by Ennor & Rosenberg (1954b), it can be made to proceed almost quantitatively from L to R (above) at pH 7·2. Reaction V is catalysed by creatine phosphokinase (CPK) and myokinase and will not proceed in the presence of CPK only since AMP is not an acceptor for the PC-CPK system (Ennor and Rosenberg, 1954b). A catalytic amount of either ATP or ADP is necessary to start the reaction, which then becomes self-accelerating.

Labelled ATP and PC were obtained either from an animal or a tissue suspension to which $NaH_2{}^{32}PO_4$ had been administered. In the former case it has been shown that all three P groups of ATP become labelled, though not to the same extent (Ennor and Rosenberg, 1954a). From the tabular representation of the reactions employed for the synthesis of specifically labelled compounds (Table 1) it may be seen that the various procedures are similar and consist mainly in the transfer of a P group from PC to either ADP or

TABLE 1

Scheme for the preparation of ^{32}P-labelled compounds.

The following symbols are used:— * for labelled P group; APPP, APP, AP, P refer to ATP, ADP, AMP, PO_4^{-3} respectively. For enzymic systems indicated, see text.

	Compound required	Reactant (and source)	Reaction	Product
(1)	AP*	AP*P*P* (animal)	III	AP*+2P*
(2)	AP*P*	AP*P*P* (animal)	I_a (or I_b)	AP*P*+P* (or gluc.6−*P)
(3)	AP*PP	AP* (1 above)+PC	V	AP*PP+Creatine
(4)	AP*P	AP*PP (3)	I	AP*P+P
(5)	AP*PP*	AP*P(4)+P*C (animal)	IV	AP*PP*+Creatine
(6)	APP*P*	AP+P*C (animal)	V	APP*P*+Creatine
(7)	APP*	APP*P* (6 above)	I	APP*+P*
(8)	APP*P	APP* (7 above)+PC	IV	APP*P+Creatine
(9)	APPP*	APP+P*C (animal)	IV	APPP*+Creatine
(10)	AP*P*P	AP*P* (2 above)+PC	IV	AP*P*P+Creatine

AMP, using CPK or CPK and myokinase respectively. In some cases myosin or apyrase was used when one or two P groups, respectively, were to be removed from ATP either before or after transphosphorylation with CPK.

Because of the similarity of the reactions it was not thought necessary to carry out all procedures, and only two will be described in detail.

Materials and Methods.

ATP was prepared from rabbit muscle as described by LePage (1945). ADP was prepared from ATP by digesting with myosin at pH 9·0, and was separated from the digest as described by Dounce, Rothstein, Beyer, Meier and Freer (1948).

Both nucleotides were finally precipitated and kept as the di-barium salts, and, when required, were converted to the Na salts, as described by Ennor and Rosenberg (1954b).

AMP was obtained as the crystalline, free acid from ATP by the action of apyrase, and purified according to Kerr (1941). It was obtained and stored as the crystalline, free acid. All solutions were made up as required and adjusted to pH 7·2 by the addition of N NaOH.

A twice recrystallized commercial preparation of creatine was used. Sodium phosphocreatine was prepared as described by Ennor and Stocken (1948b). In the initial experiments ^{32}P-labelled PC was isolated from animals injected with ^{32}P, but later it was obtained by incubation of creatine and ^{32}P with a tissue suspension as described below. This latter procedure resulted in a product with a higher specific radioactivity.

^{32}P. ^{32}P was obtained from the Atomic Energy Research Establishment, Harwell, and was used in the form of $NaH_2{}^{32}PO_4$. All samples were heated in 1 N HCl at 100° C. for 1–2 hours to convert any pyrophosphate present into orthophosphate. The samples were then adjusted to the desired pH by addition of N NaOH.

All samples contained a small amount of added "carrier" orthophosphate.

Enzymes. The methods used for the preparation of myosin, myokinase, apyrase and creatine-phosphokinase have been described in a previous communication (Ennor and Rosenberg, 1954b).

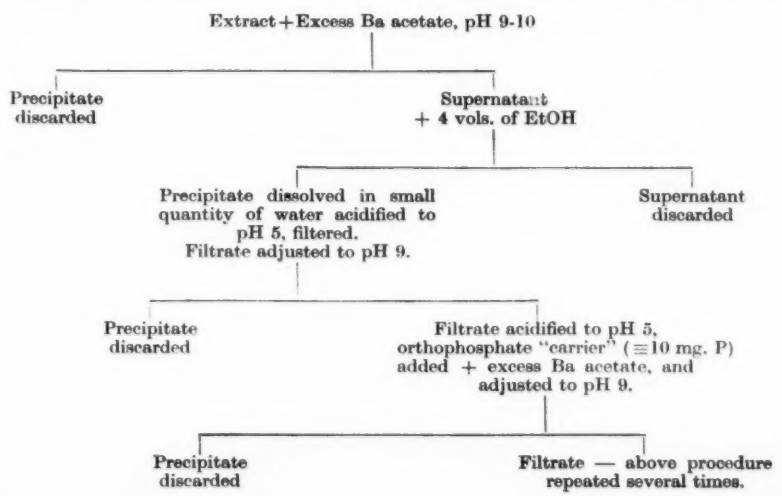
^{31}P and ^{32}P were estimated as described by Ennor and Stocken (1950) and Ennor and Rosenberg (1952), respectively. The specific radioactivity (counts/min./μg.) of the P groups of ATP and that of PC were estimated using enzymic and non-enzymic methods as described by Ennor and Rosenberg (1954a).

Creatine was estimated by the method of Ennor and Stocken (1948a).

Preparation of phosphocreatine labelled with ^{32}P.

200 gm. of muscle were taken from the hind limbs of a rabbit under nembutal anaesthesia. The muscles were immediately homogenized under an atmosphere of O_2 in a Waring blendor in 200 ml. of 0·1 *M Tris* (hydroxymethyl)-aminomethane (*Tris*) buffer, pH 7·2, containing 6·7 mMoles of creatine and 1·5 mMoles of $NaH_2{}^{32}PO_4$ containing 1 mc. of ^{32}P.

The suspension was stirred under O_2 for 3 minutes. 100 ml. of 25 p.c. (w/v) trichloroacetic acid (TCA) were then added and immediately mixed in the blendor. The precipitated protein was rapidly removed by filtration and the extract immediately adjusted to pH 7 by the addition of 5 N NaOH. PC estimations on the neutralized extract showed that approx. 1·2 mMoles of the compound were present. Separation of the PC was effected by the following procedure:


Extract + Excess Ba acetate, pH 9-10
Precipitate discarded
Supernatant + 4 vols. of EtOH
Precipitate dissolved in small quantity of water acidified to pH 5, filtered. Filtrate adjusted to pH 9.
Supernatant discarded
Precipitate discarded
Filtrate acidified to pH 5, orthophosphate "carrier" (≡10 mg. P) added + excess Ba acetate, and adjusted to pH 9.
Precipitate discarded
Filtrate — above procedure repeated several times.

Repeated precipitations with carrier $^{31}PO_4^{3-}$ were carried out in order to remove traces of highly labelled $^{32}PO_4^{3-}$. Ba^{2+} was removed from the final solution by adding Na_2SO_4 until only traces of Ba^{2+} could be detected by Na rhodizonate. The Ba_2SO_4 was then filtered off and the remaining Ba^{2+} removed by shaking the solution with Amberlite I.R.100 (Na form).

Isolation from the aqueous solution was not attempted, since the following step was carried out immediately, but the yield was 0·25 mMoles of PC with a specific radioactivity of 308 counts/min./μg. P.

Preparation of (β^{-32})ATP.

Step (1)—Preparation of (β:γ—^{32}P)ATP (Table 1, Reaction 6). 0·5 mMoles PC (Na salt) were added to the ^{32}PC above, thus yielding 0·75 mMoles of PC. The specific radioactivity of the PC-P was 103.

The following mixture was prepared: 100 ml. *M*/10 ***Tris*** pH 7·2, 60 ml. ^{32}PC solution (0·75 mMoles), 20 ml. AMP (≡0·75 mMoles), pH 7·2, CPK≡ 3·3 mg. N, myokinase≡1·0 mg. N, 2 ml. (1×10^{-3} *M*) ADP, 12 ml. (1·0 *M*) $CaCl_2$, 6 ml. water, and incubated at 37° C. The progress of the reaction (Fig. 1)

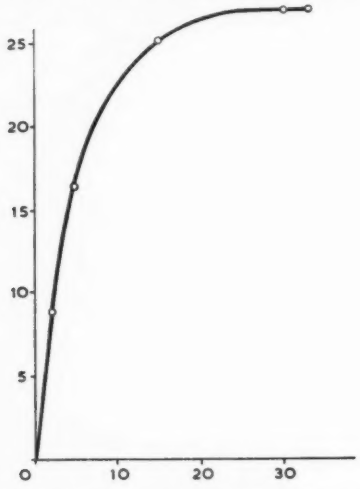


Fig. 1. Liberation of creatine during incubation of AMP with PC in the CPK-myokinase system.
Abscissa: Time, minutes. Ordinate: Microgrammes of creatine.

was followed by withdrawing small samples (0·02 ml.) and estimating the free creatine. Equilibrium was reached after 30 minutes and reaction was stopped by the addition of 50 ml. of 25 p.c. w/v TCA.

The nucleotides were isolated from the TCA filtrate by precipitation as Ba salts, which were then dissolved by the addition of glacial acetic acid and reprecipitated by the addition of 20 p.c. mercuric acetate in 2 p.c. acetic acid.

The mercury precipitate was decomposed with H_2S, and the HgS removed by filtration. The H_2S was expelled from the filtrate by aeration, and the solution adjusted to pH 7 by addition of 5 N NaOH.

Step (2)—Preparation of (β—^{32}P) ADP (Table 1, Reaction 7). The above solution was incubated with myosin in 0·025 *M* glycine buffer, pH 9·0, at 37° C. for 30 minutes. The reaction was stopped by the addition of TCA, and the ADP isolated from the filtrate as described above.

Analysis of the ADP solution showed that the yield obtained was 0·12 mMoles of ADP with a specific radioactivity in the βP of 100.

Step (3)—Preparation of β—^{32}P ATP (Table 1, Reaction 8). The following mixture: 0·12 mMoles β^{32}P ADP (above) in 40 ml. 0·05 *M Tris*, pH 7·2, 20 ml. 0·24 mMoles PC (Na salt) in 0·05 *M Tris*, pH 7·2, 7 mg. CPK (≡1 mg. N) dissolved in 16 ml. water, and 4 ml. 1·0 *M* $CaCl_2$, was incubated at 37° C.

The progress of the reaction was followed as before, and when equilibrium was reached the ATP was isolated from the deproteinized extract, as described in Step 1.

The reaction yielded 0·06 mMoles of ATP, with the following specific radioactivities: α-P—9·2, γ-P—0, β-P—104. The product was contaminated with 0·1 mMoles of orthophosphate, with a specific radioactivity of 3·8.

No further purification was carried out.

Preparation of (γ-^{32}P) ATP.

This is a one-step reaction only (Table 1, Reaction 9). Labelled phosphocreatine for this reaction was prepared as described above. Approximately 0·8 mMoles of PC were used, with a specific radioactivity of 147·0.

For the reaction 150 ml. of the solution containing 0·8 mMoles P*C and 0·6 mMoles ADP were incubated at 37° C. with 30 ml. *Tris* (0·05 *M*) pH 7·2, 12 ml. 1·0 *M* $CaCl_2$, and 8 ml. of CPK solution (≡3·2 mg. N).

The progress of the reaction was followed as described above, and equilibrium was reached after 20 min. The mixture was then incubated for a further 15 min. and then treated as described above.

The nucleotides were isolated after one single precipitation of each of the Ba and Hg salts.

The yield was 0·2 mMole of ATP with specific radioactivity of α-P—9·2, β-P—4·2, γ-P—137.

DISCUSSION.

The use of crude CPK (dialysed rabbit-muscle extract) as a synthetic tool has been suggested previously by Lehmann (1935) who considered that it might be used for phosphocreatine synthesis, using the phosphoglycerate-ATP system as a P donor. However, chemical methods (*cf.* Ennor and Stocken, 1948b) offer a more efficient method for its preparation. Synthesis of phosphocreatine

by means of a pure CPK system using ATP and creatine also seems unprofitable, due to low yields, and even if myokinase is added in an attempt to make both terminal P groups of ATP available for the reaction, the yield per mMole of ATP is still low (Ennor and Rosenberg, unpublished).

It is possible, however, that the low yields obtained may be due to denaturation of the enzyme at the high optimum pH of 10·5. On the other hand, the L→R reaction (4 above) is almost quantitative under correct conditions (Ennor and Rosenberg, 1954b), and can therefore be used for ATP synthesis.

The rather high specific radioactivity values of the α-P, both in A-P-P*-P and A-P-P-P* is an artefact arising from the methods of estimation, and due to the fact that the α-P was estimated as the difference between total (wet-ashed) and acid labile (10 min. at 100° C. in N HCl) P. If, therefore, the 10 min. hydrolysis was incomplete, some of the β-P and γ-P would have appeared as α-P.

Similarly, a specific radioactivity of approx. 4 was found in β-P in A-P-P-P*. Here β-P was estimated as the difference between apyrase labile and myosin labile P, so that if myosin did not hydrolyse 100 p.c. of the γ-P, the remainder would have been split by the apyrase and would thus be estimated as β-P. Similar results would have been achieved if traces of myokinase were present in the incubated mixture, but our preparation of CPK was myokinase-free, as shown by its inability to phosphorylate AMP.

The high specificity of the enzyme used and the simplicity of the reactions involved point to the fact that the small specific radioactivity values obtained for the α and β-P atoms must have arisen as a result of an analytical error. A specific 5′-nucleotidase would permit correct analysis and assessment of the specific radioactivity of the α-P, but this was not available.

The methods described above appear to be less complicated than that described by Lindberg and Ernster (1952), and, in addition, they offer a means of labelling any P atom in ATP.

Both Lindberg and Ernster (1952) and Hems and Bartley (1953) have assumed that the α-P was not labelled in their multi-enzyme system, although no analytical proof was given.

AP* may be prepared, as suggested in Table 1, by the dephosphorylation of uniformly labelled ATP synthesized *in vivo*. However, this compound with a high specific radioactivity may be obtained by a fermentation procedure (Albaum, Pankin and Harvill, 1953).

ATP labelled with ^{32}P in all 3P atoms was not required for the preparations described in this communication. It could be obtained, however, in the same way as ATP, but from animals injected previously with ^{32}P. Samples of such ATP have been isolated in this laboratory for analytical purposes (Ennor and Rosenberg, 1954a), and the label was found to be present in all three groups, but predominantly in β and γ. However, in long-term experiments when the

tissues were obtained 40–90 hours after ^{32}P injection, the α-P was also labelled, although to a lesser degree than were the β and γ-P atoms (Ennor and Rosenberg, unpublished). Similar observations have already been reported by Sacks and Altshuler (1942) using cat muscle, by Flock and Bollman (1944) using rat muscle, and by Sacks (1951) using rat liver.

The experiments quoted in this communication were carried out in order to show that the theory of the method was applicable experimentally. This was proven to be the case, and since the labelled substances were not required for immediate use, it was not considered worth while to carry out any further purification which could be effected by the use of anion exchange resins (*cf.* Cohn and Carter, 1950).

If a sample of pure ^{32}P-labelled PC is required, it could be obtained using labelled ATP and creatine in the reverse of reaction IV. It could, of course, be extracted from the muscles of an animal injected with ^{32}P orthophosphate, but would be contaminated with a large number of other barium-soluble ethanol-insoluble compounds, and the separation of pure PC from the mixture is extremely difficult.

If, however, pure creatine and crude ^{32}P labelled ATP are used for the preparation, the only impurities introduced are those in the ATP fraction, and they, forming insoluble Ba salts, may be easily separated from the synthesized PC which forms a soluble Ba salt.

SUMMARY.

A method for introduction of ^{32}P label into any one or more of the P atoms of ATP is described.

The method depends upon the use of enzymic systems *in vitro*.

Acknowledgments. The author is indebted to Professor A. H. Ennor, of this Department, for valuable help and discussion during the period when this work was carried out. Thanks are also due to Mr. W. A. Cleary for skilled technical assistance.

NOTE. The subject matter of this communication was given by the author in a paper to the A.N.Z.A.A.S. Conference in Canberra in January, 1954.

REFERENCES.

Albaum, H. G., Pankin, D., Harvill, E. K. (1953): Proc. Soc. exp. Biol., N.Y., 84, p. 529.
Cohn, W. E. and Carter, C. E. (1950): J. Amer. chem. Soc., 72, p. 4273.
Dounce, A. L., Rothstein, A., Beyer, G. T., Meier, R., Freer, R. M. (1948): J. biol. Chem., 174, p. 361.
Ennor, A. H. and Rosenberg, H. (1952): Biochem. J., 50, p. 524.
Ennor, A. H. and Rosenberg, H. (1954a): *Ibid.*, 56, p. 308.
Ennor, A. H. and Rosenberg, H. (1954b): *Ibid.*, 57, p. 203.
Ennor, A. H. and Stocken, L. A. (1948a): *Ibid.*, 42, p. 557.
Ennor, A. H. and Stocken, L. A. (1948b): *Ibid.*, 43, p. 190.
Ennor, A. H. and Stocken, L. A. (1950): Austral. J. exp. Biol., 28, p. 647.

Flock, E. V. and Bollman, J. L. (1944): J. biol. Chem., 152, p. 371.
Hems, R. and Bartley, W. (1953): Biochem. J., 55, p. 434.
Kerr, S. E. (1941): J. biol. Chem., 139, p. 131.
Lehmann, H. (1935): Biochem. Z., 281, p. 271.
LePage, G. A. (1945): Manometric techniques and related methods for the study of tissue metabolism, by W. W. Umbreit, R. H. Burris and J. F. Stauffer. Minneapolis: Burgess Publishing Co.
Lindberg, O. and Ernster, L. (1952): Exp. Cell Res., 3, p. 209.
Sacks, J. (1951): Arch. Biochem., 30, p. 423.
Sacks, J. and Altshuler, C. H. (1942): Amer. J. Physiol., 137, p. 750.

THE INTESTINAL ABSORPTION OF FAT, FATTY ACID AND FAT SOLUBLE DYE INTO THORACIC DUCT LYMPH IN UNANAESTHETIZED RATS[1]

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In a previous paper (Simmonds, 1954) some observations were reported which appeared inconsistent with the views of Frazer (1943) on the pathways taken by olive oil and oleic acid after intestinal absorption in the rat. It was noted that the lymph became very milky after oleic acid as well as after olive oil. There was a prolonged increase of lymph flow after both substances. The pattern of lymph flow after oleic acid was different from that after olive oil, but the total amount of extra lymph produced was about the same. In preliminary experiments, the recovery of fat-soluble dye, Sudan IV, was the same when fed in oleic acid as when fed in olive oil. These observations seemed incompatible with the passage of most of the oleic acid into the portal blood stream, Frazer's view, and it was decided to make serial measurements of fat and dye output in thoracic duct lymph after feeding dyed olive oil and dyed oleic acid to unanaesthetized rats. This paper reports the results of such experiments.

METHODS.

Male rats of an albino Wistar strain were used in all experiments. The weights varied from 220 to 320 gm.

Operative and post-operative treatment. The abdominal thoracic duct was cannulated by the method of Bollman, Cain and Grindlay (1948), a gastrostomy tube inserted as previously described (Simmonds, 1954), the wound sutured and the animal placed in a restraining cage of the type devised by Bollman (1948). This was housed in a warm box, the temperature of which was kept at about 70° F. In the first few hours after operation, 5 ml. of 0·9 p.c. sodium chloride solution were given slowly. A constant speed infusion apparatus was then connected to the gastrostomy tube and Ringer-Locke solution administered continuously at about 0·5 ml./hr. until the rat was sacrificed. At the end of each 10 hr. absorption period (see below) an extra 4–5 ml. of Ringer-Locke solution were given, representing the extra lymph production in this period. On such a regime lymph fistula rats lose weight and the daily output of lymph and urine usually exceeds by a few ml. the fluid given. However, this was considered preferable to uncontrolled variations in hydration and lymph flow with fluids *ad libitum*. The possible effects of progressive changes were controlled, as in previous experiments, by reversing the order of administration of test substances in repeated trials.

[1]This work was performed with the aid of a grant from the National Health and Medical Research Council, Canberra.

Collection of lymph and care of fistula were as previously described.

Experimental procedure. No food was given other than the dyed olive oil or oleic acid. Experiments began at 9 a.m. on the morning following operation, the rats thus being 16–20 hrs. post-operative and about 24 hours fasted. Each experimental session consisted of three periods. In the first, from 9 a.m. to 11 a.m. or later, lymph was collected to establish a baseline for lymph flow and fat output. When a steady rate of flow had been maintained for not less than an hour, 0·5 ml. dyed olive oil or oleic acid was injected by syringe into the gastrostomy tube and flushed through with about 1 ml. of Ringer-Locke solution. This marked the beginning of the second period which lasted 10 hours. Lymph flow was measured at half-hourly intervals, and fat and dye concentrations determined in the lymph collected from 0 to 1 hr., 1 to 3 hr., 3 to 6 hr. and 6 to 10 hr. The third period then followed, lasting from 9 or 10 p.m. until 9 a.m. the following morning. In a number of rats a second experimental period then commenced, the procedure being exactly the same as on the first day except that the alternative test substance was given after the baseline period.

Preparation and dose of dyed fat. Saturated solutions of Sudan IV (Coleman and Bell) in olive oil (B.P.) and oleic acid (B.P.) were prepared by adding 2 mg. dye per ml., stirring and triturating at intervals for several days then centrifuging to remove undissolved dye. The stock solutions were prepared afresh several times during the series of experiments. The dose in all cases was 0·5 ml. Corrected for the calibration error of the syringe, it was calculated that 1·50 m.eq. of fat were injected in the case of olive oil and 1·46 m.eq. in the case of oleic acid. For ease of calculating fat recoveries it was taken that 1·5 m.eq. was injected in each case.

Analytical methods. Total esterified fat in lymph was measured by the method of Stern and Shapiro (1953). It has been repeatedly shown that only a few per cent. of the total fat in lymph was unesterified, free fatty acid, whether fat or fatty acid were fed. *Dye concentrations in lymph* were measured relative to dye concentrations in the injected olive oil or oleic acid as follows: Most of the water was extracted from 1 ml. or 2 ml. aliquots of lymph, depending on the dye concentration, by shaking briefly with an equal volume of chloroform (B.P.), centrifuging and removing the dye-free watery layer. Two ml. of alcohol-ether mixture (3:1) were then added, the bulky precipitate dispersed with a glass rod and, after centrifuging, the fluid, now containing nearly all the dye, poured into a volumetric flask. A second extraction with 2 ml. of alcohol-ether removed the final traces of dyed fluid from the precipitate, leaving it white. The extracts were pooled, made up to 10 ml. with alcohol-ether and filtered. Their optical densities were measured, against alcohol-ether, in a Beckmann spectrophotometer at 520 mμ. Absorption was maximal at this wavelength for extracts of dye from lymph and dilutions of dyed fat in alcohol-ether. Dye concentration in lymph was calculated as a percentage of the concentration in the administered olive oil or oleic acid, from the dilution and optical density of the extract and the optical density of dilutions of the appropriate dyed fat in alcohol-ether. Extracts of lymph collected before fat administration did not differ appreciably in optical density from undyed alcohol-ether. Duplicate estimations of fat and dye concentrations differed by more than 3 to 4 p.c. only in the case of low fat concentrations, when 5 to 10 p.c. differences were sometimes encountered.

The rate of output of absorbed fat in the lymph was calculated from the difference between the rate of output in a given period after administration and the rate of output before fat was given, in the first experimental period referred to above. The rate and total recoveries have been expressed as a percentage of the administered dose of fat, for comparison with the corresponding values for dye.

RESULTS.

Percentage recovery of fat and dye. In a preliminary series of 9 experiments in 5 rats, mentioned previously, only dye recoveries were measured. The dye concentrations were estimated, less precisely than in the main series, by Klett visual colorimeter in extracts of serial samples collected at varied intervals in the first 7 hours and then overnight. After dyed olive oil, the mean total recovery was 19·0±2·8 p.c. of the injected dye, and after oleic acid 18·9±2·8 p.c. (mean±s.e.m.). These results compare well with those in the more detailed investigation in another 8 rats which will now be reported.

TABLE 1

Total fat and dye recoveries in thoracic duct lymph, as p.c. injected, and ratio fat: dye recovery after 0·5 ml. olive oil or oleic acid dyed with Sudan IV.

1st Day				2nd Day			
Rat No.	Fat	Dye	Ratio Fat : Dye	Rat No.	Fat	Dye	Ratio Fat : Dye
(a) after dyed olive oil							
8	77·3	24·1	3·2	16	87·2	34·3	2·5
11	64·7	16·0	4·0	12*	83·7	10·8	7·8
14	66·9	14·3	4·7	18	78·9	25·6	3·1
Mean—24 hr.	69·6	18·1	4·0		83·2	23·6	4·4
Mean—10 hr.	69·6	18·1			76·1±7·4	22·5±7·9	
(b) after dyed oleic acid							
10	56·9	16·5	3·4	7	81·3	14·8	5·5
12*	73·9	14·3	5·2	11	73·8	13·5	5·5
16	73·2	19·3	4·5	14	83·7	18·2	4·6
18	72·0	16·8	4·3				
Mean—24 hr.	69·0	16·7	4·4		79·6	15·5	5·2
Mean—10 hr.	58·8±9·4	13·0±2·8			79·6	15·0±1·6	

* See text.

The total recoveries of fat and dye in 13 experiments in 8 rats are presented in Table 1. The mean recoveries, with their standard errors, in the first 10 hours after administering dyed fat are also shown separately. With the exception of rat 12, which differed from the other 7 rats—and from another group of 6 rats given undyed fat—in the slowness of absorption of both oleic acid and olive oil, all the measurable excess fat output in the lymph and, nearly all the dye output, occurred in the first 10 hours. By the time the second experimental session began, 22 hours after the first fat injection, the fat output in the lymph was no greater than in the baseline period of the first experimental day. Mean values for the first and second day were 27·5 and 24 μeq./hr. respectively.

Table 1 shows that the amount of fat and dye recovered in the lymph on the first post-operative day was the same after oleic acid as after olive oil.

This was also true for the second post-operative day so far as fat was concerned, although a somewhat greater output of dye after olive oil was suggested. There appeared to be a consistent increase in fat recovery on the second experimental day. The mean increase was significant statistically for olive oil and oleic acid considered together ($P<0{\cdot}01$), for olive oil ($P<0{\cdot}05$) and on the borderline for oleic acid ($P<0{\cdot}1$). There were no consistent differences in the ratio of fat recovery to dye recovery attributable to the type of fat given.

The pattern of fat and dye output in the lymph. While the results in Table 1 showed that the total amount of fat and dye transported by the lymph was about the same after dyed oleic acid as after dyed olive oil, analysis of the rate of transport in successive intervals after giving dyed fat showed that absorption of dyed oleic acid was slower but more prolonged. This is illustrated by Fig. 1, in which the mean rates of output during 4 successive intervals in the first 10 hours have been plotted. The results in rat 12 have been omitted as absorption was much slower than usual in this rat, although the difference in pattern between oleic acid and olive oil absorption was still apparent. Fig. 1 also suggests that, while the output of absorbed fat and of dye varied in much the same way, fat output increased at first more rapidly than dye output. The increase in output of fat on the second experimental day seemed to occur

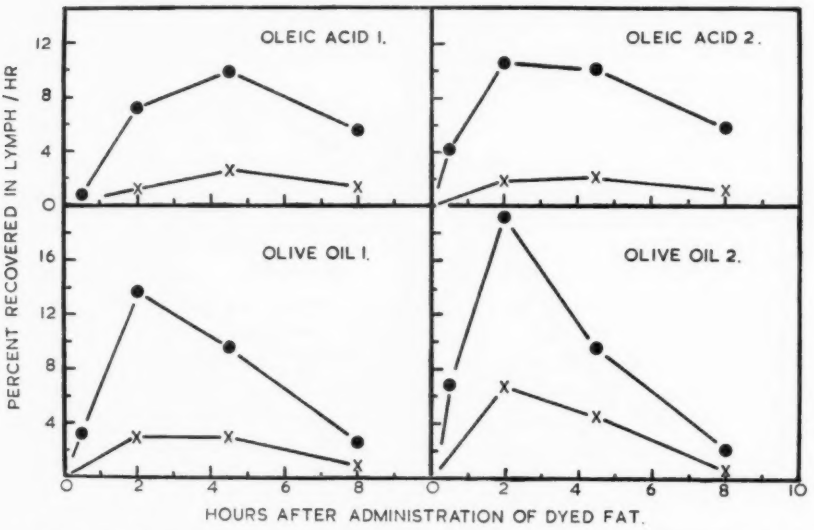

Fig. 1. The patterns of output in the lymph of dye (crosses), and excess fat (filled circles), as p.c. of injected dose per hour. The mean outputs for successive periods of 1, 2, 3 and 4 hrs. are placed at the mid-point of the period in each diagram. Average results for the first post-operative day (left hand side of diagram) are for 3 rats with dyed oleic acid and 3 with dyed olive oil; for the second post-operative day for 3 and 2 rats respectively.

almost entirely in the early stages, from 0 to 3 hours after administration. Thus, with oleic acid on the second day the output of fat was greater than that on the first day by 2·6 p.c. of the injected dose from 0 to 1 hour, by 6·6 p.c. from 1 to 3 hours, and by 1·9 p.c. from 3 to 10 hours. For olive oil on the second day the values were +4·6, +11·1 and −2·2 p.c. respectively.

The differences suggested above were tested statistically by analysis of variance of the individual results in the various periods in 4 rats, Nos. 11, 14, 16 and 18, which received both fat treatments. In two of these animals oleic acid was given first and in the other two olive oil. These rats were representative of those studied in the present series, except rat 12, in respect to fat and dye outputs, lymph flows, etc. They also gave results similar to those in rats used for other purposes in which less complete data were available. Analysis revealed significant differences between the pattern of fat and dye output after oleic acid and that after olive oil ($P<0·01$ for the appropriate interaction). When the intervals 0 to 1, 1 to 3, 3 to 6, and 6 to 10 hours were compared separately, there was a significant difference ($P<0·05$) in the period 0 to 1 hour in fat output on the two days and with the two types of fat. Analysis of variance of the ratios of fat output to dye output in the periods of 1 to 3 hours and 3 to 6 hours showed significant differences consistent with those suggested by Fig. 1; for example between periods, the ratio from 1 to 3 hours being higher than from 3 to 5 hours.

Finally, it may be mentioned that comparisons of lymph flow, fat and dye concentrations showed the more abrupt rise and fall of lymph flow with olive oil encountered in previous experiments (Simmonds, 1954), and a relation between lymph flow and fat output similar to that described by Borgstrom and Laurell (1953). These points will be considered at a later date when experiments on the relation between increased lymph flow and fat absorption are presented.

DISCUSSION.

In these experiments the amount of the fat-soluble dye, Sudan IV, and of fat which passed into the lymphatics after intestinal absorption was of the same order after dyed oleic acid was given as after dyed olive oil. In both cases much more fat than dye was recovered. The smaller recovery of dye cannot be attributed to incomplete extraction from lymph (see Methods) nor to fading of the dye. Examination of the faeces and inspection of the tissues at post-mortem did not reveal dye staining. Qualitative observations reported in the literature on the absorption of dye and fat after feeding dyed fat have been conflicting. Gage and Fish (1924) found dyed fat in the intestinal lymphatics of cats and rats after feeding Sudan-stained palmitic and stearic acids. Frazer's (1943) results suggested that little Sudan IV or fat appeared in the lymph of

rats after dyed oleic acid, while dyed olive oil passed readily into the lymph. Verzar and McDougall (1936) reported that fat, but no dye, was seen in the intestinal lymphatics in rats after milk, cream or olive oil stained with Sudan-red, although both dye and fat appeared in rabbits. Quantitatively, Auld and Needham's (1951) experiments in a patient with chylothorax supported Frazer's views. They recovered from the pleural fluid 20 p.c. of Sudan III fed in olive oil, but little or none after dyed oleic acid.

The similarity in the amounts of esterified fats recovered after olive oil and oleic acid in the rats is consistent with the greater part of the literature on the recovery of labelled and unlabelled long-chain fatty acids in the lymph, and with published data on the composition of lymph after feeding these substances (e.g., Verzar and McDougall, 1936; Freeman and Ivy, 1935; Bloom, Chaikoff, Reinhardt, Entenman and Dauben, 1950; Bollmann, Flock, Cain and Grindlay, 1950; Borgström, 1951). Recently, Frazer (1952) has suggested that high recoveries of labelled, long-chain saturated fatty acids in the lymph when fed with unlabelled triglyceride have been erroneously interpreted, and that the tracer acids were carried in with emulsified triglycerides. It is difficult to reconcile this suggestion with results such as those of Borgström (1952), who found that the absorption of C_{14}-labelled palmitic acid from the intestine and its recovery in intestinal lymph were much the same whether transesterified with corn oil, dissolved in corn oil, or dissolved in free fatty acids obtained by saponification of corn oil. Specific activities suggested that, when dissolved in corn oil, labelled palmitic acid was incorporated into lymph fat more rapidly at first than was the carrier triglyceride.

Little emphasis is placed on the actual values calculated for percentage of administered fat recovered in lymph, although these agree quite well with results in the literature on labelled fat absorption. The possible inaccuracies in a balance method, without chemically labelled fat, should not invalidate comparison of the results after olive oil with those after oleic acid. Also, since only esterified fat was measured by the analytical method used, these experiments, like many earlier ones in the literature, emphasize the ease and efficiency with which large amounts of long-chain acids may be resynthesized to neutral fat and other esters and despatched in the lymph. Frazer (1952) has suggested that the inconsistency between his earlier experiments (Frazer, 1943) in which the chylomicrons in the systemic blood increased very much less after oleic acid than after olive oil, and those of Tidwell (1950), who found no difference, might have been due to fine emulsification in the latter's experiments. However, fat is very rapidly removed from the blood when infused in the form of chylomicrons in fatty lymph (Morris, 1955). It is, therefore, possible that a more gradual rise and fall in the rate of fat transport in the lymph would give rise to a flatter systemic chylomicron curve, irrespective of the total amount absorbed,

as Borgström (1952) has already suggested. The differences in pattern of fat output in the lymph between olive oil and oleic acid feeding experiments have been described above. They are consistent with those demonstrated by Borgström (1952) with labelled fats and due probably to differences in gastric emptying. With 1 ml. of fat or fatty acid, which seems to have been the dose used by Frazer (1943), differences might be more striking than with the smaller dose (0·5 ml. or less) used by the other workers mentioned. Nevertheless, this would not explain the differences in portal chylomicron curves described by Frazer, nor would lymphatic connections with the portal vein (Job, 1918) explain how particulate fat appeared in the portal blood stream more readily after oleic acid.

The demonstration of a significant increase in fat output on the second experimental day was useful in analysis of the results, but its biological significance cannot be assessed from the present data. Displacement of fat remaining in the intestinal epithelium from the first experimental session is perhaps the most likely explanation. Improvement of fat absorption on the second post-operative day or a facultative increase in fat absorption (Tuba and Robinson, 1953) are other possibilities.

SUMMARY.

Olive oil and oleic acid, in doses of 0·5 ml. containing dissolved Sudan IV, were fed to unanaesthetized rats with plastic cannulae in their abdominal thoracic ducts.

The recovery of dye in thoracic duct lymph was usually 15–20 p.c. fed and the mean recovery after oleic acid did not differ significantly from that after olive oil. The excess output of fat in the lymph was the same in each case and equivalent to 70 to 80 p.c. of the fat fed. There were differences in the pattern of dye and fat output, both of which increased more rapidly to a higher maximum value and decreased more abruptly after olive oil than after oleic acid.

The inconsistencies between these findings and some which have been adduced in support of the Partition Hypothesis are briefly discussed.

Acknowledgment. I wish to thank Miss M. Woodward for technical assistance.

Addendum.

Shortly after this paper had been submitted for publication, Bergström, Blomstrand and Borgström (1954) reported that the percentage of absorbed label recovered in thoracic duct lymph in rats was the same after feeding oleic acid containing the (1-C_{14}) labelled variety as after triolein synthesized from (1-C_{14}) oleic acid. Thus quantitative data on the recovery of fat soluble dye, unlabelled fat, and, most conclusively, of isotopically labelled fat show that the pathway for absorbed oleic acid is the same as that for olive oil.

Bergström, S., Blomstrand, R. and Borgström, B. (1954): Biochem. J., 58, p. 600.

REFERENCES.

Auld, W. H. R. and Needham, C. D. (1951): Lancet, 1, p. 991.
Bloom, B., Chaikoff, I. L., Reinhardt, W. O., Entenman, C. and Dauben, W. G. (1950): J. biol. Chem., 184, p. 1.
Bollman, J. L. (1948): J. lab. clin. Med., 33, p. 1348.
Bollman, J. L., Cain, J. C. and Grindlay, J. H. (1948): *Ibid.*, 33, p. 1349.
Bollman, J. L., Flock, E. V., Cain, J. C. and Grindlay, J. H. (1950): Amer. J. Physiol., 163, p. 41.
Borgström, B. (1951): Acta chem. Scand., 5, p. 643.
Borgström, B. (1952): Acta physiol. Scand., 25, p. 291.
Borgström, B. and Laurell, C.-B. (1953): *Ibid.*, 29, p. 264.
Frazer, A. C. (1943): J. Physiol., 102, p. 306.
Frazer, A. C. (1952): Biochem. Soc. Symp., 9, p. 1 (Cambridge University Press).
Freeman, S. and Ivy, A. C. (1935): Amer. J. Physiol., 114, p. 132.
Gage, S. H. and Fish, P. A. (1924): Amer. J. Anat., 34, p. 1.
Job, T. T. (1918): *Ibid.*, 24, p. 467.
Morris, B. (1955): Austral. J. exp. Biol., 54, p. 763.
Simmonds, W. J. (1954): *Ibid.*, 32, p. 385.
Stern, I. and Shapiro, B. (1953): J. clin. Path., 6, p. 158.
Tidwell, H. C. (1950): J. biol. Chem., 182, p. 405.
Tuba, J. and Robinson, M. I. (1953): *Ibid.*, 203, p. 947.
Verzar, F. and McDougall, E. J. (1936): Absorption from the intestine. Longmans, Green & Co., London.

ANAEROBIOSIS WITH IRON WOOL

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Several techniques for the removal of oxygen from sealed containers are described in the Manual of Methods for the Pure Culture Study of Bacteria (1952). Of these, two appear to be used most widely in the study of anaerobic bacteria: the alkaline pyrogallol method and the McIntosh and Fildes method.

The alkaline pyrogallol technique has two serious defects. Carbon dioxide is absorbed if the alkaline solution used is a hydroxide, and carbon dioxide is now generally regarded as being indispensable to the growth of bacteria. Absorption of carbon dioxide may be avoided by the use of alkaline carbonate solutions, but whether carbonate or hydroxide solutions are used, appreciable quantities of carbon monoxide are evolved (Nicol, 1929). Carbon monoxide is a respiratory poison.

The McIntosh and Fildes technique is widely used in routine anaerobic work. Apart from the expensive apparatus involved, a large amount of residual hydrogen gas remains in the jar after the oxygen has been removed. Hydrogen has been shown to inhibit nitrogen fixation (Burris and Wilson, 1945) and to inhibit the stripping of hydrogen from pyruvic acid by *Clostridium butyricum* (Mortenson and Wilson, 1951). Further, if oxygen gains entrance to the jar during incubation it is not effectively removed, and sensitive anaerobes fail to grow.

A method of oxygen removal which does not possess the defects discussed is therefore desirable. The inclusion of metallic iron in liquid media as a means of promoting the growth of anaerobes has been developed by Spray (1936) and by Hayward and Miles (1943). A technique is presented here which extends the use of metallic iron to the elimination of oxygen from the atmosphere of sealed containers.

EXPERIMENTAL.

Oxygen removal.

One gm. lots of commercial iron wool (grade 0) were treated by immersion for 30 seconds in solutions of copper sulphate at different concentrations which were acidified to pH 1·5–2 with sulphuric acid. A wetting agent, Lissapol N.300*, was added to the solution at a concentration of 0·25 p.c. The iron was then drained of excess solution and tested for rate of oxygen uptake in normal air.

*Product of I.C.I.

50 ml. of 0·25–0·5 p.c. copper sulphate solution gave the most effective activation of the iron. At 22° C. 1 gm. iron wool absorbed approximately 1 ml. oxygen per minute when treated with 50 ml. 0·25 or 0·5 p.c. copper sulphate solution; 0·7 ml. per minute with 0·125 p.c. solution, and 0·7 ml. per minute with 1 p.c. solution. All solutions contained 0·25 p.c. Lissapol and were adjusted to pH 2. When the wetting agent was omitted from the solution, the rate of oxidation of the iron was substantially reduced, but when the copper sulphate was omitted the rate of oxidation was very markedly reduced, the iron taking up to 10 minutes to absorb 1 ml. oxygen.

Iron wool treated with copper sulphate solution in the manner described becomes irregularly spotted with metallic copper, the couples so formed being subject to rapid oxidation. If the copper is plated too evenly over the surface of the iron the rate of oxygen uptake is slower. It also decreases with decreasing partial pressure of oxygen in the container, so that in practice, with a three litre jar at 28° C., 10 gm. of treated iron takes four to five hours to decolourize the methylene blue indicator. At 37° C. the indicator may be decolourized in 2½ hours.

Manometric readings showed a reduction in pressure equivalent to 150 mm. of mercury in the vessel containing the iron, and methylene blue indicator solution was decolourized. Thus the oxygen is removed without the evolution of any substantial quantity of hydrogen.

Carbon dioxide absorption by iron.

When present in equal volume with oxygen, carbon dioxide is absorbed by the treated iron in equimolar proportions, but it is not absorbed to any appreciable extent in the absence of oxygen. Thus carbon dioxide can not easily be supplied by addition in gaseous form; it may, however, be supplied by the addition to the container of suitable saturated carbonate solutions. With these the carbon dioxide vapour pressure within the jar rises to the normal for the particular solution as soon as the oxygen is removed. Conditions then become suitable for the growth of anaerobes.

The carbon dioxide vapour pressures of various saturated carbonate, bicarbonate and mixed carbonate solutions at 28° C. were determined by the Petten-

System	Carbon dioxide (v.p. in mm Hg)	P.c. Carbon dioxide
Na_2CO_3	1·1	0·14
$Na_2CO_3+NaHCO_3$	4·5	0·6
$MgCO_3+NaHCO_3$	10·6	1·4
$NaCl+NaHCO_3$	34	4·5
$CaCO_3+NaHCO_3$	38	5·0
$NaHCO_3$	78	10·3

*Product of I.C.I.

kofer method. Where two salts are specified, equal weights of each were mixed, and excess solid was present in all cases. Those solutions which gave a steady vapour pressure over a period of eight hours are reported below.

The methylene blue indicator.

The standard three-solution methylene blue indicator (Manual for the Pure Culture Study of Bacteria, 1952) deteriorates on keeping. The single-solution indicator of Ulrich and Larsen (1948) also deteriorated rapidly, probably due to polymerization of the methylene blue.

The modified indicator described has proved satisfactory in this laboratory, and the solutions have remained useful for over one year. Two stock solutions are prepared: (1) 3 ml. of 0·5 p.c. aqueous methylene blue diluted to 100 ml. with distilled water; (2) 0·5 gm. glucose dissolved in 100 ml. distilled water, a crystal of thymol (approx. 50 mg.) added and the solution adjusted to pH 10 with 0·1 molar Na_2CO_3 in 0·1 molar $NaHCO_3$.

Store the solutions in the dark. The indicator is prepared by mixing these two solutions so that a light to medium blue colour results. The tube of indicator may be added to the anaerobic jar cold, and will remain coloured until the oxygen has been reduced to less than 0·05 atmospheres.

The Anaerobic Method.

The following technique is suitable for the growth of anaerobes in a three-litre jar.

Mould 10 gm. of commercial iron wool, grade 0 or grade 1, into a loose pad to fit a petri dish lid. Dip each pad in 500 ml. of an acid solution of 0·25–0·5 p.c. copper sulphate in 0·25 p.c. wetting agent such as Lissapol or Tween 80. This solution should be acidified with sulphuric acid to a pH of 1·5–2. Drain excess solution from the pad and place the pad in a petri dish wherever convenient in the jar. Place a suitable carbonate solution and a tube of the methylene blue indicator (see Methods) in the jar and seal.

RESULTS.

Anaerobic bacteria were grown in jars containing iron wool on solid and in liquid media with the following results:

Growth on the surface of solid media. Viable counts of 24-hour broth cultures of *Clostridium butyricum* diluted in sterile tap water and plated on potato dextrose agar, were compared using the McIntosh and Fildes anaerobic method and the iron wool method. Both systems were supplied with 1·4 p.c. carbon dioxide from the $MgCO_3/NaHCO_3$ mixture. Counting was done by the dropping method (Miles and Misra, 1938).

A typical count is given below:

	Colonies per drop mean of eight drops	Viable cells per ml. broth culture
McIntosh and Fildes Jar:	19·1±1·8	955,000
Iron wool Jar:	19·5±1·7	975,000

No significant differences were evident in the counts obtained by the two techniques. However, the McIntosh and Fildes jars frequently failed owing to small air leaks, whilst the iron wool jars gave consistent and effective anaerobiosis.

Growth in liquid media. During the course of nitrogen fixation studies on *Clostridium butyricum*, this organism was incubated in shallow liquid layers 2–3 mm. deep in standard petri dishes for periods of 10–14 days. The iron wool produced and maintained satisfactory anaerobic conditions throughout these experiments.

DISCUSSION.

The successful use of this technique depends on the maintenance of a large surface area of wet activated iron in contact with the atmosphere of the jar. Copper sulphate has been found most successful as an activator, but 1 p.c. acetic acid solution may be substituted. This treatment will give good removal of oxygen, but a considerable amount of hydrogen is produced. For ordinary routine work there is little need to weigh out the materials to be used, although the quantities given have been found to be effective and convenient. Hydrogen production is negligible provided that a large quantity of the acid copper sulphate solution is not allowed to remain with the iron during incubation.

Carbon dioxide should be added by the method described, or colony formation and growth will be seriously affected. There has been no evidence of inhibition due to any cause other than absorption of carbon dioxide by the iron.

The technique is economical, rapid, and easy to use. Evacuation apparatus, gas cylinders and electricity are not required. It is adaptable in that by altering the proportion of iron to container volume, the time taken to remove the oxygen can be reduced. Anaerobic conditions may also be obtained rapidly by sweeping out with an inert gas, the iron being used as a scavenger. Oxygen which gains entrance through minor leaks in the apparatus is removed while metallic iron remains.

Anaerobic bacteria of the butyric group have been grown using this method, and for these it has proved a useful and reliable method of anaerobiosis.

SUMMARY.

Oxygen is removed from sealed containers by the oxidation of activated iron wool. Strictly anaerobic bacteria may be grown in such containers, either on the surface of solid media or in shallow liquid layers. Carbon dioxide must be added to the containers.

Also described are (a) a means of obtaining a range of reasonably constant carbon dioxide vapour pressures, and (b) an improved methylene blue indicator of anaerobiosis.

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Addendum.

It is worthy of mention that on two occasions during routine anaerobic work, ammonia was evolved from the iron wool pads. All attempts, here and elsewhere, have failed to repeat this ammonia production. The possibility remains that the ammonia observed was catalytically produced on the surface of the iron by the reduction of molecular nitrogen. In any anaerobic experiments where the presence of ammonia might be critical it would be advisable to watch this aspect.

REFERENCES.

Burris, R. H. and Wilson, P. W. (1945): Ann. Rev. Biochem., 14, p. 685.
Hayward, Nancy J. and Miles, A. A. (1943): Lancet, 244, p. 641.
Miles, A. A. and Misra, S. S. (1938): J. Hyg. Camb., 38, p. 732.
Mortenson, L. E. and Wilson, P. W. (1951): J. Bact., 62, p. 513.
Nicol, H. (1929): Biochem. J., 23, p. 324.
Society of American Bacteriologists (1952): Manual of Methods for the Pure Culture Study of Bacteria, 10th ed., Biotech. Publications, Geneva, N.Y.
Spray, R. S. (1936): J. Bact., 32, p. 135.

THE SODIUM THIOSULPHATE SPACE AS AN ESTIMATE OF EXTRACELLULAR FLUID VOLUME IN RABBITS

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At present, no entirely satisfactory methods of estimating extracellular fluid volume are available for routine physiological or clinical studies.

The first important difficulty is that our concept of extracellular fluid cannot be defined by reference either to anatomical boundaries or to the distribution of any normal body solute so that the finding of any "true" value for its volume can never be expected. The additional notions that some of the extracellular water exists as a "transcellular" phase (Edelman *et al.*, 1952a), and some as a slowly equilibrating connective tissue phase (Nichols, 1953), support the idea of a rapidly equilibrating fraction or "physiological volume" of the extracellular water, but leave the problem of its measurement unsolved.

The equilibrium volume of distribution of some injected tracer substance can be used as a parameter of extracellular fluid volume if collateral evidence from tissue studies can establish its predominantly extracellular character. Since thiocyanate was first introduced for this purpose (Crandall and Anderson, 1934), a great variety of substances has been tried and, in turn, discredited for the reasons detailed by Levitt and Gaudino (1950). The only two to meet with much support in recent years are inulin and sucrose. But to approximate an equilibrium distribution of inulin requires a constant infusion for at least 5 hours (Gaudino, 1948), and for sucrose a calibrated infusion for about 3 hours is needed (Deane, 1951). Both methods involve urine collection and seem cumbersome and unsuitable for routine use either with patients or with small animals—situations where accurate urine recovery cannot be guaranteed.

The use of thiosulphate to estimate the extracellular space seems to deserve a wider trial than it has yet received. Gilman (1946) first showed that the thiosulphate ion had, in dogs, an equilibrium volume of distribution in the range of the extracellular fluid volume and its plasma clearance after equilibrium was proportional to its concentration. Schwartz (1950) and Cardozo and Edelman (1952) confirmed these findings in both dogs and man. Kowalski and Rutstein (1952) found that, unlike thiocyanate, thiosulphate was not bound

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to plasma proteins, but did, however, slowly enter red blood cells. Cardozo and Edelman (1952) showed that this penetration of red cells was insignificant under 70 minutes, but in this period there was a slow extra-renal loss of thiosulphate, presumably due to metabolism. This extra-renal loss probably occurs mainly in muscle (Nichols, 1953), and in anuric dogs follows an exponential rate (Gilman, 1946). Cardozo and Edelman (1952) pointed out that when there is linearity of the total clearance extrapolation will correct for such slow losses across cell membranes. They also showed that the volume of distribution of thiosulphate, measured by a single-injection extrapolation method, gave a satisfactory estimate of changes in extracellular fluid volume in patients.

Thiosulphate, then, is predominantly an extracellular ion.

The present study shows that the total plasma clearance of thiosulphate in the rabbit is also linear. The volume of distribution of thiosulphate obtained by the single-injection extrapolation method is proposed and discussed as a suitable estimate of the extracellular space in this species.

MATERIALS AND METHODS.

Five healthy adult cross-bred rabbits ranging in weight from 1·9 to 2·4 kg. were used in seven studies—two animals were studied a second time. There was no restriction of their food or fluid intake prior to the experiments.

Blood samples of 1–2 ml. were drawn from a marginal ear vein, each by a fresh venepuncture with a 23-gauge Luer needle fitted to a dry all-glass 2 ml. syringe.

An all-glass 10 ml. syringe and a plastic tubing connection were calibrated for delivery of a thiosulphate solution. This was an approximately 5 p.c. aqueous solution of commercial hydrated sodium thiosulphate ($Na_2S_2O_3 \cdot 5\ H_2O$). The contents of two 10 ml. ampoules[2] were pooled and a part used for injection, a part for accurate determination of its concentration.

A stop-watch was used to record the infusion and sampling times to the nearest five seconds.

First a blood sample was drawn for serum blank estimation: the needle was left in the vein. Through this needle 10 ml. of the thiosulphate solution were infused over 5 minutes. With too rapid injections (1 minute or less) extrapolation does not correct for large pre-equilibrium losses due to serum concentrations above the initial extrapolated concentration (Raisz, 1953). An equilibration period of at least another 5 minutes was allowed because, after slow injections, there is usually a linear rate of fall of serum thiosulphate concentration after 10 minutes (Raisz, 1953).

Five serial blood samples were then taken from the opposite ear at intervals of about 5–10 minutes.

Thiosulphate in the serum samples and in the infused solution was estimated by a micromodification (Cardozo and Edelman, 1952) of the iodate method of Gilman (Newman, Gilman and Philips, 1946).

In 9 preliminary trials our recovery from serum by this method averaged 98·8 p.c. with a range of 94–103 p.c. All determinations were done in duplicate which, overall, were reproducible within an average range of 7 parts per 1,000.

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[2]Drug Houses of Australia Ltd.

Each mean serum concentration was plotted semilogarithmically against the mid-time of its collection period and linear plots were obtained (Fig. 1).

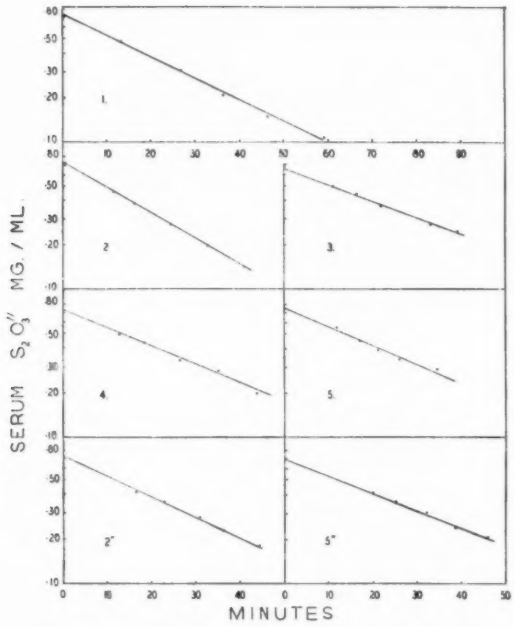

Fig. 1. Venous disappearance curves of thiosulphate in rabbits. First experiments 1–5: second experiments 2″ and 5″.

Each regression line was calculated by the method of least squares. Extrapolation of this line gives the hypothetical equilibrium concentration at zero time. The ratio of the injected dose to this equilibrium concentration was taken as the volume of distribution.

RESULTS.

Experimental.

The calculated volumes of distribution of thiosulphate are shown in Table 1. For comparison they are also expressed as percentages of the body weights, accompanied in brackets by the variance due to regression for each estimate.

The values in the seven studies ranged from 17·5–23·5 p.c. with a mean of 20·08 p.c. of body weight. The differences between the first and second results for rabbits Nos. 2 and 5 were respectively −2·2 p.c. and +2·1 p.c. of body weight.

TABLE 1

Volume of distribution of thiosulphate in rabbits

Rabbits	Date	Weight gm.	Thiosulphate space	
			ml.	% b. wt.
1	29/4/53	2208	432·4	19·58 (1·29)
2	4/5/53	2235	440·1	19·69 (0·26)
3	6/5/53	2106	441·3	20·95 (0·75)
4	13/5/53	2345	422·6	18·02 (1·29)
5	18/5/53	1946	415·7	21·36 (0·57)
2*	27/5/53	2404	421·0	17·51 (0·43)
5*	25/5/53	1973	463·0	23·47 (0·98)
Mean				20·08 (0·796)
Variance of the seven estimates				4·257

* Second experiments.

Variance analysis.

The variance of the estimate of the thiosulphate space in any one rabbit was calculated from the corresponding regression analysis and the difference between the variances so obtained was not statistically significant. This suggests that the experimental conditions and the accuracy of the chemical methods were constant from trial to trial.

These variances of the thiosulphate spaces, due to regression alone, had a mean of 0·796 (with 21 d.f.).

The variance between the seven different thiosulphate spaces was 4·257 (with 6 d.f.) and this is significantly greater ($p<0·001$) than 0·796. Thus the major part of the variance is between experiments rather than within experiments—that is, there are probably real differences between the rabbits.

DISCUSSION.

As to the differences between rabbits there are two factors to consider.

One, which has been appreciated for several years, is that the amount of water in a body is inversely related to body fat. The total body water may vary from 45–70 p.c. of body weight in adult individuals (Edelman *et. al.*, 1952b) so that we might reasonably expect the extracellular fluid volume to differ by up to 5–10 p.c. of body weight within a population.

In rabbit No. 2, which gained 169 gm. in three weeks, it seems likely that body fat was laid down because there was only a slight fall of 19 ml. in the thiosulphate space, but this had fallen by 2·1 p.c. relative to body weight.

The normal day to day variation in body water content (Lowe, 1953) is another factor less often considered. In the rabbit it may be as much as 3 p.c. of body weight (Fowler, 1955), and one would expect the extracellular fluid to be largely concerned in such fluctuations.

This is of obvious importance when evaluating any method by reproducibility and may be relevant to rabbit No. 5, which gained only 27 gm., yet its thiosulphate space had risen by 47 ml. to a volume which was 2·2 p.c. greater relative to body weight.

The evaluation of any method must depend partly on a comparison of results from different workers. As yet, relatively few have used thiosulphate as a measure of extracellular fluid volume. The published results with its use are here collated for the first time as shown in Table 2.

TABLE 2

Previous estimates of thiosulphate space

Workers	Method	Experimental subjects	Thiosulphate space % b. wt.
Gilman, Philips and Koelle (1946)	Blood and urine collection	4 dogs	Range: 20·4—24·2 Mean: 22·0
Schwartz (1950)	Constant infusion and urine collection.	4 dogs	Range: not stated. Mean: 17
		2 men	15·7 and 19·5
Cardozo and Edelman (1952)	Single slow injection and extrapolation.	7 dogs	Range: 20·3—28·1 Mean: 24·4
		6 men	Range: 14·9—17·8 Mean: 16·6
Raisz (1953)	Single slow injection and extrapolation.	3 dogs	Range: 20—24·72 Mean: 22·05
Nichols (1953)	Tissue analyses after single injections.	8 dogs	Mean: 21% (at zero time)

Our own results in the rabbit agree well with other estimates of the extracellular space in this species as well as with those listed above for dogs and humans. In nephrectomized rabbits the simultaneous inulin and sucrose spaces are slightly less than 20 p.c. of the body weight as estimated by Deane (1951) from the studies of Kruhoffer. But these were made under pentobarbital anaesthesia, so their significance is uncertain. However, Manery and Haege (1941) measured the chloride space in five rabbits with the isotope Cl^{38} and obtained a mean of 21 p.c. with a range of 17–26 p.c. of body weight.

We feel that sufficient evidence has been given to support a plea for more extensive trials of thiosulphate in the estimation of extracellular fluid volume. In the normal animal our figures confirm that the simpler single-injection extrapolation method gives results in the same range as do more elaborate techniques using either thiosulphate, sucrose, inulin or chloride.

SUMMARY.

Thiosulphate spaces were measured in rabbits by a single-injection extrapolation method.

The mean of these determinations was a volume equivalent to 20 p.c. of body weight.

Evidence is presented that this is an acceptable yardstick for measuring extracellular fluid volume in the rabbit.

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REFERENCES.

Cardozo, R. H. and Edelman, I. S. (1952): J. clin. Invest., 31, p. 280.
Crandall, L. W., Jnr. and Anderson, M. X. (1934): Amer. J. digest. Dis., 1, p. 126.
Deane, N., Schreiner, G. E. and Robertson, J. S. (1951): J. clin. Invest., 31, p. 1463.
Edelman, I. S., Olney, J. M., James, A. H., Brooks, L. and Moore, F. D. (1952): Science, 115, p. 447.
Edelman, I. S., Haley, H. B., Schloerb, P. R., Sheldon, D. B., Friis-Hansen, B. J., Stoll, G. and Moore, F. D. (1952b): Surg. Gyn. Obst., 95, p. 1.
Fowler, R., Jnr. (1955): Austral. Ann. Med., in press.
Gaudino, M., Schwartz, I. L. and Levitt, M. F. (1948): Proc. Soc. exp. Biol. Med., 68, p. 507.
Gilman, A., Philips, F. S. and Koelle, E. S. (1946): Amer. J. Physiol., 146, p. 348.
Kowalski, H. J. and Rutstein, D. D. (1952): J. clin. Invest., 31, p. 370.
Levitt, M. F. and Gaudino, M. (1950): Amer. J. Med., 9, p. 208.
Lowe, T. E. (1953): Austral. Ann. Med., 2, p. 136.
Manery, J. F. and Haege, L. F. (1951): Amer. J. Physiol., 134, p. 83.
Newman, E. V., Gilman, A. and Philips, F. S. (1946): Bull. Johns Hopkins Hosp., 79, p. 229.
Nichols, G., Jnr., Nichols, N., Weil, W. B. and Wallace, W. M. (1953): J. clin. Invest., 32, p. 1299.
Raisz, L. G., Young, M. K., Jnr. and Stinson, I. J. (1953): Amer. J. Physiol., 174, p. 72.
Schwartz, I. L. (1950): *Ibid.*, 160, p. 526.

THE PRODUCTION OF VACCINIA HAEMAGGLUTININ BY THE CHORIOALLANTOIC MEMBRANE OF EMBRYONATED EGGS

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(Accepted for publication 20th September, 1954.)

The production of a haemagglutinin (VHA) by vaccinia-infected cells was first noted by Burnet, who found that an emulsion of vaccinia-infected chorioallantoic membranes would agglutinate a suspension of fowl cells.

Production of a haemagglutinin has also been noted in the skin of vaccinia-infected rabbits by Stone and Burnet (1946). It was shown (Burnet and Stone, 1946) that the haemagglutinin produced is not the virus particle itself but is, nevertheless, a specific substance produced only by vaccinia-infected cells.

By differential centrifugation it was shown that infective virus may be removed from the supernatant fluid without loss of VHA titre. Further, the VHA may be removed by adsorption with fowl red blood cells without loss of virus. The haemagglutination reaction is reversible by the addition of specific antiserum.

Nagler and Clark (1943) found that only 50 p.c. of fowls provide cells susceptible to agglutination.

The present work was carried out to determine the time of first appearance and the rate of production of the haemagglutinin in infected cells in relation to the rise in infectivity, and the effect of varying the size of the inoculum on the production of VHA.

An investigation was also carried out of suitable methods for the large scale production of vaccinia haemagglutinin for further physico-chemical investigation of the haemagglutinin.

The results obtained suggest that there is an initial delay in the production of detectable haemagglutinin until virus is released from infected cells in the first growth step. From this point the haemagglutinin titre rises logarithmically for 12–24 hours, with a gradual tapering off in the production rate after this period.

The time of first appearance of detectable VHA depends on the infective dose used and, in general, the amount produced seems to be a function of the number of cells infected at that time.

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[1]Carden Fellow in Cancer Research, Anti-Cancer Council of Victoria.

Materials and Methods.

The vaccinia strain "Gillard", derived from an American commercial vaccine lymph and cultured on the chorioallantoic membrane, was used throughout as stock.

Eggs which had been incubated at 37·5° C. for 11–12 days were inoculated on the chorioallantoic membrane with varying numbers of infectious particles of vaccinia virus prepared by making broth dilutions of stock virus.

Following inoculation, the eggs were incubated at 37° C.

At intervals following inoculation, the eggs were harvested and the chorioallantoic or allantoic membranes removed and placed in a piston-barrel type tissue mincer.

This consisted of a metal chamber into which a closely-fitting piston could be forced by a hand press. The working capacity of the mincer was 1·5 ml. and both container and piston were sterilized by boiling before use. Repeated checks, using stained smears of the emulsified material, were made from time to time to ensure that all cells of the membrane were being fragmented.

It has been found by Anderson (1954) that the infective titre of a C-A membrane is reduced less by such a method than by grinding with silica in a pestle and mortar.

To the membrane in the mincer was added 0·5 ml. of heated normal rabbit serum as diluent. The membrane was ground twice in the mincer, then the resulting suspension was centrifuged at 2500 r.p.m. for 3 minutes to remove the largest cellular debris.

Normal rabbit serum, heated to 62° C. for 15 minutes, was used as a diluent for both the emulsification of the membrane and subsequent haemagglutination titrations. Heated rabbit serum has been found to prevent non-specific haemagglutination of susceptible fowl red blood cells by lipoids (Burnet and Stone, 1942). It was used as a 1 p.c. solution in normal saline.

Allantoic fluid when used was harvested before removing the membranes. The fluid was then spun at 2500 r.p.m. for 3 minutes to remove red blood cells. Using the resulting supernate, titrations for infectivity and for haemagglutinin were carried out.

Infectivity titrations were made on the chorioallantois, six embryos being normally used for each tenfold dilution. The weighted average pock count was determined as described by Burnet and Faris (1943).

Haemagglutinin titrations were made by making serial twofold dilutions of tissue extract in the rabbit serum saline.

An equal volume (0·25 ml.) of 0·1 p.c. R.B.C. from a "susceptible" fowl was added. The racks were placed in an incubator at 37° C. and shaken after 10–15 minutes. The cells were then allowed to settle undisturbed for an hour. Readings were based on the pattern made by the deposited cells on the bottom of the tube and the end point being taken as that of partial agglutination (+). Readings were facilitated by the use of ×2 magnification.

It was found that, by using suspensions of 0·1 p.c. red cells instead of 1 p.c. red cells, the titre of haemagglutinin detectable was increased tenfold.

The infecting titre and haemagglutinin titres were then calculated as respective titres per membrane and the $\log_{10}$ of these titres used in both Tables and graphs.

RESULTS.

Comparison of efficiency of methods for handling membranes.

An investigation of the VHA yields from membranes emulsified in various ways was made with a view to obtaining maximal yields for large scale production of VHA.

The results may be seen in Table 1. In each case membranes infected for 48 hours with 3×10^2 infectious particles were used.

TABLE 1

Method of Emulsification	Log_{10} titre V.H.A. per membrane
Grinding + silica with Pestle and mortar	2·40
	2·32
Mincing for 25 mins. in modified Waring blendor	2·40
	2·40
Grinding in piston—barrel mincer	3·38
	3·38
Grinding with Teflon grinder	3·28
	3·30

As may be seen from this Table, membranes ground with pestle and mortar or minced in a Waring blendor showed a lower VHA titre than similar membranes emulsified in the piston-barrel type of tissue mincer or the Teflon tissue grinder.

The results described in the body of this paper were obtained using the piston-barrel type mincer. This was preferred as it consisted of two simple metal parts which allowed easy sterilization by boiling. This was of importance when making infectivity titrations of the membrane being emulsified.

For large scale production of emulsified membranes for investigation of the physico-chemical properties of VHA, it was found more convenient to use the Teflon type of tissue grinder, which was capable of handling several membranes at a time.

General production curve of VHA.

Using an inoculum of 3×10^2 infectious particles, the haemagglutinin titres of membranes at varying times following infection were estimated.

As can be seen from Fig. 1, no detectable amounts of haemagglutinin were produced until 20–22 hours following inoculation. At this point a sharp rise in titre occurred. The titre increased logarithmically at a uniform rate with no obvious steps until 36 hours, when there was a flattening out of the curve. The VHA showed a slow increase in the titre from this point up to 72 hours, when variations in individual eggs became more marked. Correlated with this variation in the titre of VHA was a similar wide variation in the naked eye appearance of the membranes. Those membranes which were grey, oedematous, opaque and friable with confluent growth over the surface of the membrane yielded higher titres of VHA than those membranes of more normal thickness and hydration, with only part of their surface covered with vaccinia lesions.

Effects of variation in inoculum.

In Fig. 2 may be seen the curves obtained when inocula of 7×10^4 and 3×10^2 infectious particles were used.

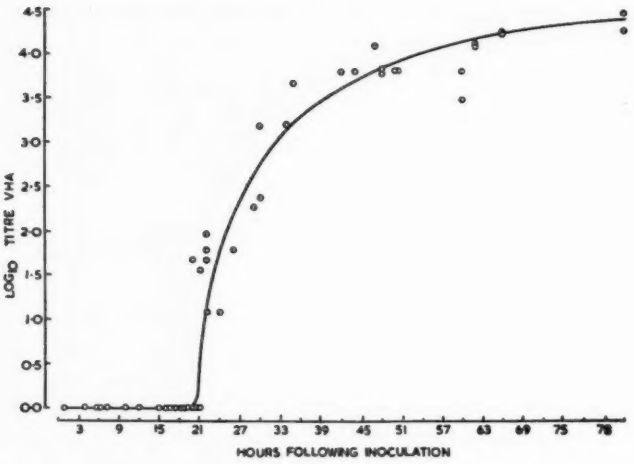

Fig. 1. General production curve of V.H.A. Initial inoculum 3×10^2 infectious particles.

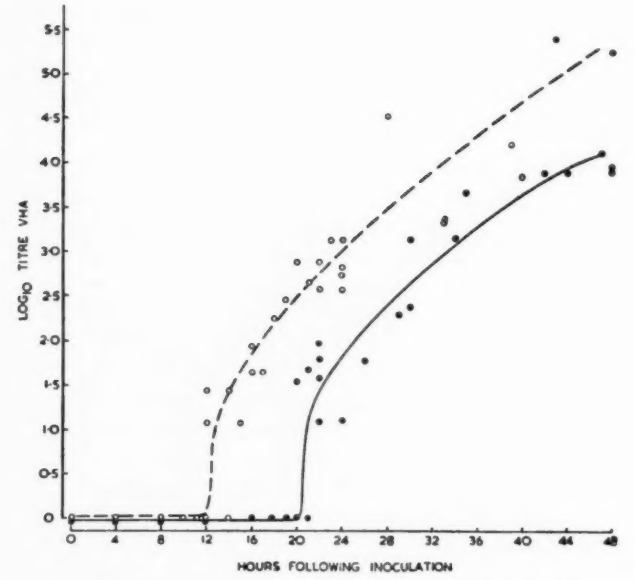

Fig. 2. Production curve of V.H.A. with variation in size of infecting inoculum.
○ = Inoculum of 7×10^4 infectious particles.
● = Inoculum of 3×10^2 infectious particles.

In each case the general characteristics of the curve are similar—a latent period, followed by a rapid production of VHA, succeeded by a gradual and variable production of VHA in the final stage. However, with increasing numbers of infectious particles in the inoculum, VHA appeared earlier and reached higher titres. With an initial inoculum of 10^6 infectious particles, appearance of detectable VHA occurred after about 9 hours. Results were variable and the majority of the eggs died within the first 12–18 hours, making further titrations impossible.

Correlation of VHA production and infectivity.

As can be seen from Fig. 3, the production of VHA ran parallel with the rise in infectivity titre in the chorioallantoic membrane. In this experiment an infectivity inoculum of 7×10^4 particles was used. This relationship is emphasized when the two growth curves are shown together as in Fig. 3.

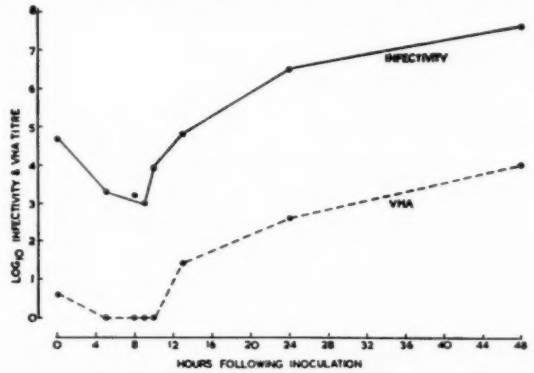

Fig. 3. Infectivity and V.H.A. growth curves following chorioallantoic inoculation. Initial inoculum—7×10^4 infectious particles.

Further, as seen in Fig. 2, when a smaller inoculum (3×10^2) was used, there was a delay in the appearance of detectable VHA until 20–22 hours following infection which corresponds approximately to two 8–9 hour growth cycles.

Production of VHA following allantoic inoculation.

When vaccinia virus was introduced into the allantoic cavity, the production of vaccinia haemagglutinin in the allantoic membrane followed closely the pattern of that for the chorioallantoic membrane. However, there were two differences; (a) there was a longer lag period, (b) the titres of VHA produced were lower than those in the chorioallantoic membrane.

Fig. 4 shows the curves obtained when comparable inocula are introduced into these two systems.

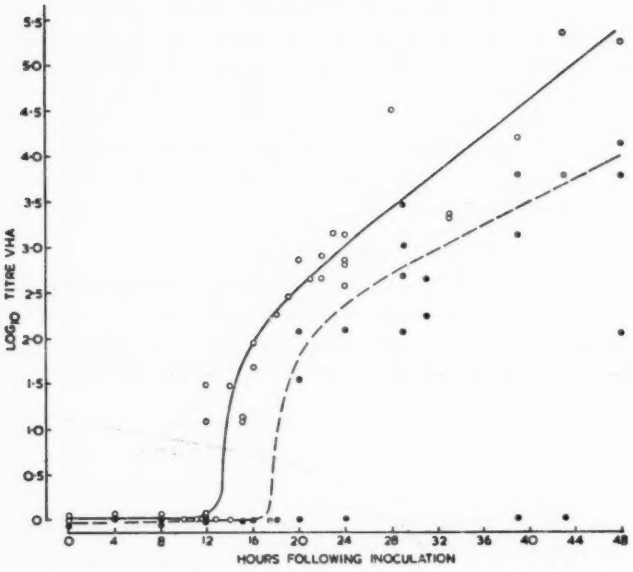

Fig. 4. V.H.A. growth curves following chorioallantoic and sub-allantoic inoculation.
○ = Inoculum of 7×10^4 particles on chorioallantoic membrane. Titre in chorioallantoic membrane.
⊙ = Inoculum of 7×10^4 particles into allantoic cavity. Titre in allantoic membrane.
● = Inoculum of 7×10^4 particles into allantoic cavity. Titre of allantoic fluid.

When the allantoic fluid itself was titrated for haemagglutinating activity, the results were very irregular. Some eggs showed quite high titres of VHA, others showed a complete absence of haemagglutinin.

Fig. 4 also shows the variability of results obtained with allantoic fluid.

Production of VHA in other sites in the egg. Following both chorioallantoic inoculation and allantoic cavity inoculation, titrations of VHA activity were made on various organs of the embryo. In all cases, no detectable VHA was found up to 72 hours following inoculation.

Possible inhibitor substances to haemagglutination in the CA membrane.

In view of the possibility that the failure to detect VHA, before the appearance of newly-produced infective virus, might be due to the action of inhibitory substances present in the tissue or cells of the chorioallantoic membrane, an attempt was made to demonstrate their presence.

Incubation at 37° C. for several hours of a mixture of emulsified infected membranes with an excess of emulsified normal membranes was carried out. The infected membranes were chosen early in the growth cycle of VHA and

were expected to yield a low titre of VHA. When the dilution factor had been corrected, no fall in the resultant VHA titre was found when compared with control membranes.

No evidence for an inhibitor substance was found.

DISCUSSION.

Haemagglutination of fowl red cells by emulsions of vaccinia-infected tissues has been shown to be both specific and unrelated to the vaccinia particle itself (Burnet and Stone, 1946).

However, as may be seen from the above Figures, the production of haemagglutinin bears a direct temporal and quantitative relationship to the number of infectious vaccinia particles being produced. With inocula of 7×10^4 infectious particles on the chorioallantoic membrane, the production of detectable haemagglutinin follows closely upon the rise in titre of the infectious vaccinia particles occurring at the end of the first growth cycle. From this point the curves are roughly parallel, the ratio of infective titre/haemagglutinin titre being of the order of 10^3–$10^4/_1$.

Further, with increases in the size of the infecting inoculum, the lag period for the appearance of detectable haemagglutinin progressively shortens. This is presumably related to the number of infected host cells, a minimal number being required before detection of haemagglutinin is possible.

It would appear that the cells lining the allantoic cavity are not as suitable for the multiplication of vaccinia virus as those of the chorioallantoic membrane. There is both a longer delay in the production of detectable haemagglutinin and a lower final titre produced when identical inocula are used.

This investigation was undertaken primarily to determine the most suitable site in the egg for VHA production on a large scale basis and the best method for emulsifying the infected tissue.

It would appear that the chorioallantoic membrane is the tissue most suited for the production of high titres of VHA.

An initial inoculum of approximately 10^4 infectious particles and an incubation period of 48 hours were finally chosen as the combination giving reasonably high titre material with a reasonable number of viable eggs.

Membrane emulsification with a Teflon type grinder was found to be the most efficient of various methods tried.

SUMMARY.

The growth curve of vaccinia haemagglutinin in the embryonated egg has been investigated.

There is a lag period after inoculation, followed then by a period of rapid production of haemagglutinin with a final slowing down of production rate.

The length of the lag period and the final titres of haemagglutinin produced are dependent on the number of infectious particles used in the inoculum and the type of cell infected.

There is a parallel relationship between the titres of infectious particles and the titres of haemagglutinin produced by the infected cells.

The presence of haemagglutinin inhibiting substances could not be demonstated.

Standard procedure for production of VHA on the chorioallantoic membrane is described.

Acknowledgments. I am indebted to Sir Macfarlane Burnet, F.R.S., and Dr. S. G. Anderson for their helpful advice and criticism, and to Miss M. Cook for her assistance with the diagrams.

REFERENCES.

Anderson, S. G. (1954): Personal communication.
Burnet, F. M. and Faris, D. (1942): J. Bact., 44, p. 241.
Burnet, F. M. and Stone, J. D. (1946): Austral. J. exp. Biol., 24, p. 1.
Nagler, F. P. O. and Clark, E. (1942): *Ibid.*, 21, p. 103.
Stone, J. D. and Burnet, F. M. (1946): *Ibid.*, 24, p. 9.

MULTIPLICATION OF VIRUSES IN MOSQUITOES FOLLOWING FEEDING AND INJECTION INTO THE BODY CAVITY[1]

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The virus of Murray Valley encephalitis (MVE) was shown to be transmitted to chickens by four species of culicine mosquito and, prior to the occurrence of transmission, the virus underwent a biological cycle in the mosquito (McLean, 1953a). All eleven culicine species which were tested carried large amounts of virus 10 days or more after virus was fed. The findings suggested that virus first multiplied in the gut, was liberated into haemocoele where it underwent further multiplication, and thus it was carried to the salivary glands.

Merrill and Tenbroeck (1935) showed that eastern equine encephalomyelitis (EEE) virus multiplied in *Aedes aegypti* if it were injected into the body cavity of the mosquito or if the gut were punctured after an infective blood meal, but the virus did not multiply after simple feeding to the mosquito. Hurlbut (1951) showed that Japanese B encephalitis (JBE) virus multiplied in culicine mosquitoes after injection into the body cavity, and that mosquitoes transmitted virus if they bit suckling mice two days or more after injection. The movement of JBE virus through mosquitoes was not recorded.

The present work was undertaken to obtain further data on the multiplication of MVE virus in the mosquito and its movement through the mosquito. Virus was introduced into the gut by feeding, directly into the body cavity by injection, or indirectly into the body cavity by puncture of the gut immediately after a meal of blood and virus. MVE virus multiplied readily in culicines after injection, and transmission by mosquito bite occurred 3 days or more after injection.

Anopheline mosquitoes have not been incriminated as vectors of encephalitis viruses (Reeves and Hammon, 1946). Previous work (McLean, 1953a) has shown that MVE virus was not carried after being fed to *Anopheles annulipes*, the only anopheline examined. Evidence will be presented in this paper to show that if MVE virus were introduced into the body cavity of *Anopheles annulipes* the virus underwent multiplication at a slower rate than the multiplication of virus after injection into culicines.

[1]This work was aided by a grant from the National Health and Medical Research Council, Canberra.

MATERIALS AND METHODS.

Most mosquitoes used in these experiments were obtained wild from Corowa and Urana, about 200 miles north-east of Melbourne, between January and April, 1954. No epidemic of MVE has occurred in these regions since 1951 (Anderson *et al.*, 1954). Employing a plastic sucking tube, introduced to us by Dr. W. C. Reeves, of the University of California, *Culex annulirostris* and *Anopheles annulipes* (Figs. 1 and 2) were captured whilst feeding on humans during the evening, or from rabbit burrows during the day. Mr. K. Myers, of C.S.I.R.O. Wildlife Survey Section, Albury, also provided mosquitoes captured in specially constructed traps which employed rabbits as live bait (Myers, 1954). *Aedes queenslandis* (previously referred to as *A. occidentalis*) (McLean, 1953a) was obtained near Melbourne.

Mosquitoes were handled in the laboratory as described previously (McLean, 1953a). However, it was found that *A. annulipes* and *C. annulirostris* survived injection or gut puncture in greater numbers if they were held at 80° F. for at least one day prior to manipulation. This routine was, therefore, adopted in handling later consignments of mosquitoes. Deaths due to trauma usually occurred within 24 hours after injection.

Dissection of mosquitoes. One or two mosquitoes were killed with chloroform. The following anatomical units were dissected out, ground separately and extracted with 5 p.c. rabbit serum saline; salivary glands, thorax, stomach and hindgut, ovaries, legs. The stomach and hindgut will be referred to subsequently as "gut". Alundum was used to grind the thoraces and legs, but it was not required to grind the other anatomical units. In the present work, the salivary glands were dissected free of the head. Otherwise the technique of dissection was the same as described previously (McLean, 1953a).

Viruses. MVE virus, seed number 78409, was used as an emulsion of mouse brain, 0·05 ml. of which contained $10^{6.3}$ egg infective doses (EID). CF_5 virus, isolated by Mr. E. L. French, of these laboratories (Reeves *et al.*, 1954), was used as a suspension of infected chorioallantoic membranes in the 6th or 7th passage. 0·05 ml. of this material contained about $10^{2.0}$ EID. Influenza virus strain MEL, seed number 49244, was stored as infected allantoic fluid. Neurovaccinia, seed number 51056, and herpes simplex, strain HFE, seed number 42028, were used as infected chorioallantoic membrane suspensions. All were sealed in glass capillaries and stored in a dry ice refrigerator at −70° C.

Titration of virus. Suspensions of mosquitoes or their tissues or of seed virus were made in 5 p.c. rabbit serum saline. The titre of these suspensions was determined by chorioallantoic inoculation of 11-day chick embryos, for MVE, CF_5, neurovaccinia and herpes. Influenza virus was titrated by suballantoic inoculation of 11-day embryos.

Injection of mosquitoes. Pyrex glass tubing ¼-in. diameter was drawn out quickly in a hot gas coal flame fed with compressed air. The capillary was then drawn out in a small flame of the pilot light of a Bunsen burner so that a capillary about 10μ diameter was produced.

Mosquitoes were anaesthetized with CO_2 supplied from a cylinder. They were placed under a 3-in. diameter hand lens. The capillary pipette, which had been filled with virus suspension, was inserted into the thorax between the legs and wings and the fluid was injected. By weighing mosquitoes before and after injection, it was found that approximately 0·001 ml. was injected into each mosquito. The mosquitoes were then placed in a cage, in which they rapidly recovered from the anaesthetic.

To puncture the abdomen, we anaesthetized freshly gorged mosquitoes with CO_2 and laid them laterally on a sheet of cork. Puncture of the gut was performed with a capillary similar to that used for injecting mosquitoes.

RESULTS.

MVE virus in Culex annulirostris.

Five batches of *C. annulirostris* mosquitoes were injected with MVE virus, approximately $10^{3.0}$ EID per insect. One or two mosquitoes were removed at intervals after injection, they were dissected and pools were made of similar anatomical units. The pools were titrated for virus (Table 1 and Fig. 3).

TABLE 1

MVE virus in C. annulirostris *at intervals after injection.*

Anatomical unit	Hours after virus was injected					
	Zero	6	12	24	48	72
Salivary glands	0*	0	0	2·3	3·7	3·8
Thorax	2·7+	2·7	1·4	4·3	5·5	5·0
Gut	0	0	0	2·3	3·3	3·7
Ovaries	0	—	—	0	1·4	1·3
Legs	2·3	1·4	0	3·0	3·8	3·9

+ Geometric mean log EID of MVE virus in each anatomical unit pool.
* No virus was detected, i.e. $<10^{1.3}$ EID per unit.
— Not tested.

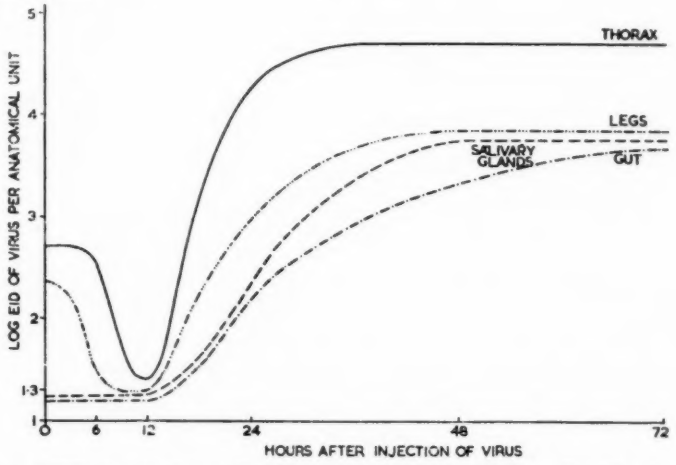

Fig. 3. MVE virus in *C. annulirostris* at intervals after injection. Smooth curves derived from data in Table 1.

Three batches of mosquitoes were fed MVE virus in defibrinated rabbit blood. Each mosquito engorged approximately $10^{3.0}$ EID of virus. The virus content of each anatomical unit was determined at intervals after the infective blood meal (Table 2 and Fig. 4).

TABLE 2

MVE virus in C. annulirostris *at intervals after feeding.*

Anatomical unit	Days after virus was fed					
	Zero	1	2	3	4	6
Salivary glands	0*	—	0	1·8	2·1	3·5
Thorax	0	0	0	2·7	4·1	4·7
Gut	2·7+	0	1·3	2·1	4·0	4·5
Ovaries	0	—	—	—	2·1	1·8
Legs	0	0	0	2·7	2·5	2·8

+ Geometric mean log EID of MVE virus in each anatomical unit pool.
* No virus was detected, i.e. $<10^{1\cdot3}$ EID per unit.
— Not tested.

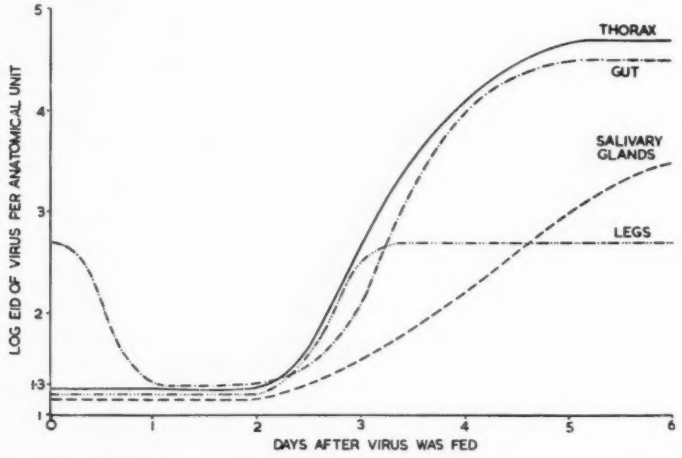

Fig. 4. MVE virus in *C. annulirostris* at intervals after feeding. Smooth curves derived from data in Table 2.

In the thorax, the final concentration of virus was approximately the same whether virus was injected into or fed to the mosquito. However, the titre had become maximal on the second day after injection, whereas it was not maximal until the sixth day after feeding. In both cases, the overall increase in amount of virus was about one hundred-fold. Minimal amounts of virus were recovered from the thorax 12 hours after injection, but thereafter a steady increase in the amount of virus occurred until the second day.

In the salivary glands virus was not present at 0, 6, 12 hours after injection, but was present at 24 hours. The maximum concentration was reached by 48 hours. After virus was fed, virus was not detected before the third day, and maximum amounts were reached by the sixth day.

In the gut, virus first appeared 24 hours after injection and maximum amounts were reached by the third day. After feeding, virus was present in the gut almost immediately after a blood meal. If blood were expelled during dissection of the gut no virus was detected in the gut wall. By the second day virus was absent or was present only in small amounts, but by the sixth day maximum amounts were recorded.

The ovaries contained only a low concentration of virus whether virus was injected into or fed to the mosquito.

The legs contained virus immediately after injection, since the tissue spaces in the legs are in communication with the body cavity. Virus was not detected 12 hours later, but by 24 hours some was recovered and the maximum concentration was attained by 48 hours. Virus fed to mosquitoes was not detected in the legs before the third day, after which it remained at approximately the same titre.

These results show that MVE virus multiplied after injection into *C. annulirostris*, as well as after virus was fed. An eclipse phase, during which little or no infective virus was recovered from any anatomical unit, has been demonstrated after both modes of introduction of virus. By comparing the titre of virus in the sites of inoculation (Fig. 5), we find that in the thorax after injection minimum amounts were recovered at 12 hours, but in the gut after feeding minimum amounts were recovered at 24 and 48 hours. Thereafter a steady increase in amount of virus occurred in both anatomical units, but the virus content of the thorax after injection increased more rapidly and attained a higher final titre than the gut after feeding.

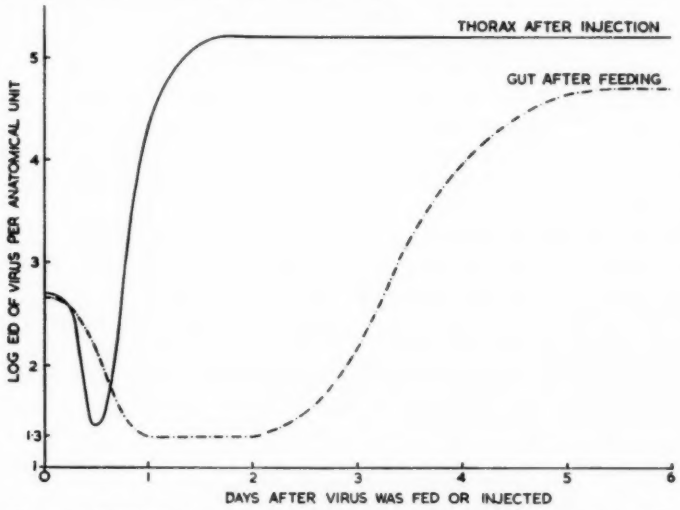

Fig. 5. MVE virus content of thorax after injection and gut after feeding.

The technique of injection caused virus to be introduced into the haemocoele as shown by its presence in the legs immediately after injection. This brought it into contact with large numbers of muscle, ectodermal and other non-enteral cells. Groups of muscle fibres are separated only by a system of spaces which constitute the haemocoele. After a short eclipse phase in the first 12 hours during which virus was probably in the non-infective multiplying phase inside the non-enteral cells, infective virus was liberated into the haemocoelic fluid until after the second day. At the time of injection, virus was conveyed to other anatomical units, in particular the salivary glands and the gut. In both these situations multiplication of virus took place, and virus was first detected after 24 hours, increasing to a maximum on the second day.

When virus was fed to the culicine mosquito, the progress of infection appeared to follow this sequence: immediately after the infective blood meal virus was present only in the lumen of the gut. During the first and second days virus was predominantly in the non-infective form inside the tissues which comprise the gut. On the third day infective virus was liberated into the coelom, where it infected large numbers of non-enteral cells. Virus also reached the salivary glands and multiplied there, maximum titres being reached by the sixth day.

On several occasions the virus content of whole mosquitoes was determined. Virus was detected on the twenty-fourth day after injection at a titre of $10^{4.3}$ EID per insect, and on the forty-eighth day at a titre of $10^{3.8}$ EID. Therefore virus appeared to persist in mosquitoes for approximately 7 weeks after injection.

Immediately after injection of $10^{3.0}$ EID of MVE virus and on the first, second, third and seventh days, mosquitoes were fixed with alcoholic Bouin solution and stained with haematoxylin and eosin. No histological change which could be attributed to the multiplication of virus was detected in any of these sections. The lack of production of lesions in mosquito tissues as a result of multiplication of MVE virus is analogous to the growth of mumps virus in amniotic or allantoic cells of chick embryos without causing changes detected histologically (Watson, 1952).

Two groups of mosquitoes were injected with approximately 10 EID of virus per insect. Whole mosquitoes were killed 3 days or more after injection and their virus contents were estimated. In each case virus had multiplied to a titre of $10^{4.6}$ EID per mosquito or greater. One mosquito transmitted virus when it bit a young chicken 7 days after the mosquito had been injected.

Two groups of mosquitoes were injected with $10^{-0.5}$ EID of virus per insect. In one group, virus was detected on the tenth and fourteenth days at a titre of $10^{4.7}$ and $10^{4.6}$ EID per mosquito respectively; in the other group virus was not detected on the ninth day after injection.

Thus, the 50 p.c. infectivity dose of MVE virus for mosquitoes by injection

is slightly less than one EID. Approximately the same amount of virus will cause infection of mosquitoes after injection as will cause encephalitis in suckling mice or viraemia in fledgling chickens (McLean, 1953b).

MVE virus in Aedes queenslandis.

In the early stages of this work *A. queenslandis* mosquitoes were injected with MVE virus. Results obtained were in close agreement with these obtained for *C. annulirostris.*

Transmisison of MVE virus to chickens.

Mosquitoes were injected with approximately $10^{3.0}$ EID of MVE virus. At intervals, mosquitoes were encouraged to bite chickens less than one week old. Those mosquitoes which bit the chickens were ground and their virus contents were estimated. The chickens were bled 24 and 48 hours after being bitten and the virus content of the blood was determined. If viraemia developed in the chicken 24 and 48 hours later, we considered that transmission had occurred. The results are shown in Table 3.

It is apparent that transmission of virus occurred when mosquitoes bit chickens between 3 and 28 days after mosquitoes were injected, but transmission did not occur on the first or second days.

TABLE 3

Transmission of MVE virus from C. annulirostris *to chicken.*

Number of mosquitoes	Days after mosquito was injected	Transmission of virus	Log EID virus in mosquito pool
1	1	$\frac{0^*}{2}$	5·3,+ 4·7§
1	2	$\frac{0}{1}$	5·2§
1 or 2	3 to 28	$\frac{4}{4}$	4·1 to 5·5§

* Numerator: Number of experiments in which virus was transmitted by mosquito bite. Denominator: Number of experiments performed.
+ Log EID virus 13 days after mosquito was injected.
§ Log EID virus immediately after mosquito had fed on chicken.

If results in Table 1 and Table 3 are compared, it would appear that the presence of virus in the salivary glands was necessary for 24 to 48 hours before virus was excreted in the saliva, whence it could be injected into the tissues of the chicken during the mosquito bite. This principle also probably applies in the case of virus being fed to mosquitoes. In this case, virus was present in considerable amount for about 2 days before the mosquito transmitted virus by biting a chicken on the seventh day after an infective blood meal.

MVE virus in Anopheles annulipes.

Previous work has shown that MVE virus did not multiply after being fed to *Anopheles annulipes* (McLean, 1953a), and further observations on the present season's mosquitoes have confirmed this.

On six occasions approximately $10^{3.0}$ EID of virus were injected into anopheline mosquitoes and pairs of specimens were killed at intervals and dissected. The virus content of pools of anatomical units was then determined and the results are shown in Table 4 and Fig. 6.

TABLE 4

MVE virus in Anopheles annulipes *at intervals after injection.*

Anatomical unit	Days after virus was injected							
	Zero	1	2	3	4	6	10	14
Salivary glands	0*	0	0	0	0	2·1	2·9	3·9
Thorax	3·0+	2·8	1·8	3·6	4·0	4·4	4·6	4·5
Gut	0	0	0	0	—	0	0	1·7
Legs	1·8	1·8	0	1·7	1·3	2·7	2·7	2·7

+ Geometrical mean log EID of MVE virus in each anatomical unit pool.
* No virus was detected, i.e. $<10^{1.3}$ EID per unit.
— Not tested.

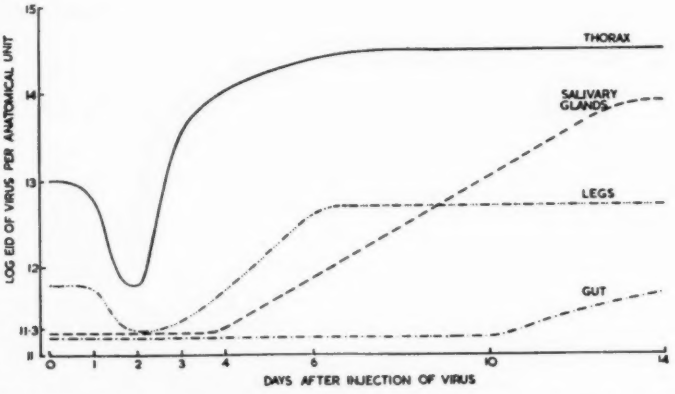

Fig. 6. MVE virus in *An. annulipes* at intervals after injection. Smooth curves derived from data in Table 4.

In the thorax the virus content diminished somewhat during the first two days. The amount of virus increased after the third day to a maximum by the sixth day, and persisted at this level thereafter. In the salivary glands virus was first detected on the sixth day and increased until between the tenth

and fourteenth days, after which it remained constant. In the gut virus was detected only occasionally, and, when it was present, the amount was less than $10^{2.0}$ EID per unit. Virus in the ovaries did not increase beyond $10^{2.0}$ EID per unit. In the legs small amounts of virus were detected immediately after inoculations, virus was not detected on the second day but the amount increased between the fourth and the sixth days, after which it remained approximately constant.

Virus appeared to multiply if it were introduced by injection directly into the body cavity. The cycle of multiplication of virus in Anopheles non-enteral tissue took 3 days, whereas less than 24 hours was required in the case of culicine tissue. Multiplication of virus occurred in the salivary glands where high titres were attained after the sixth day. Presumably, virus would be transmitted if mosquitoes bit chickens after the tenth day. Despite large quantities of virus circulating through the mosquito from the fourth day after injection onwards, practically no virus was ever recovered from the gut. This suggests that the tissues of and immediately surrounding the gut were incapable of supporting the multiplication of virus. This finding is in complete accord with the observation that no MVE virus was ever detected for more than two days after virus was fed to *Anopheles annulipes* (McLean, 1953a).

CF_5 virus in C. annulirostris *and* A. queenslandis.

A suspension of CF_5 virus which contained between $10^{2.0}$ and $10^{3.0}$ EID per ml. was fed to mosquitoes and the virus content of whole mosquitoes was determined at subsequent intervals. On four occasions out of thirteen virus was recovered from mosquitoes 14 days or more after virus was fed. Virus was not recovered before the 14th day. The presence of virus was not demonstrated well on primary isolation on the chorioallantois, but some pocks were produced on passage of these membranes. Thus, CF_5 virus multiplied hardly at all after being fed to two species of culicine mosquito.

TABLE 5

CF_5 virus in mosquitoes after injection or feeding and gut puncture.

Route of administration	Days after administration of virus						
	Zero	1	2	3	4	6	10
Injection	0*	2·7+	4·7	5·1	5·0	5·2	5·0
Feeding and gut puncture	0	1·7	4·9	5·0	—	3·8	—

+ Geometric mean log EID of virus per mosquito pool.
* No virus detected, i.e. $<10^{1.3}$ EID per mosquito.
— Not tested.

If the abdomen of mosquitoes which had recently fed on virus were punctured with a fine glass capillary, considerable amounts of virus were detected

in whole mosquitoes on the second or third day afterwards (Table 5). The presence of virus was never demonstrated immediately after the blood meal, and a very small amount was present on the first day.

Three batches of *A. queenslandis*, 2 batches of *C. annulirostris* and 1 batch of *Anopheles annulipes* were injected with CF_5 virus suspension. Virus was not detected immediately after injection, small amounts were found at 24 hours and large amounts were present at 48 hours and thereafter. In three cases these mosquito suspensions were used for injection of other mosquitoes, and in each case virus multiplied after injection, reaching a titre of about $10^{5.0}$ EID per mosquito. Virus was present in salivary glands on the second and subsequent days after injection (Table 6). In the first six days after virus was injected, there was no evidence that virus had multiplied in the gut.

TABLE 6

Localisation of CF_5 virus after injection in C. annulirostris.

Anatomical unit	Days after virus was injected					
	Zero	1	2	3	4	6
Salivary glands	0*	0	2·1+	4·8	0	2·8
Thorax	0	2·0	4·3	5·3	4·5	4·7
Gut	0	0	—	0	0	1·3
Legs	0	0	3·3	0	2·6	2·7

+ Geometric mean log EID of virus per anatomical unit.
* No virus was detected, i.e. $<10^{1.3}$ EID per unit.
— Not tested.

CF_5 virus multiplied readily in mosquitoes if it were brought directly into contact with the non-enteral tissues either by gut puncture or by injection. However, if virus were fed to the mosquito, multiplication of virus occurred only occasionally and was detected in small amounts 14 to 21 days after feeding. The time characteristics of CF_5 virus multiplication resembled closely the sequence of events after MVE virus was injected in culicine mosquitoes, except that, in the case of CF_5, no virus was recovered immediately after gut puncture or injection on account of the very small amount of virus which was administered and the relative insensitivity of the technique used to detect the presence of virus in samples of test material.

Other viruses in mosquitoes.

Concentrated suspensions of virus of influenza A strain MEL were fed to *A. queenslandis*. Neurovaccinia virus was fed to and injected into *C. annulirostris* and *A. queenslandis*, and that of herpes simplex was fed to or injected into *C. annulirostris*. In no case was any virus detected from 3 days up to 21 days after administration of $10^{3.0}$ to $10^{4.0}$ EID of any of these viruses. Feeding virus

of either influenza or neurovaccinia to mosquitoes did not interfere with the multiplication of MVE virus introduced at the same time or up to 7 days afterwards. Thus, viruses which are not normally transmitted by mosquitoes did not multiply in mosquitoes.

Fig. 1. *Culex annulirostris.*

Fig. 2. *Anopheles annulipes.*

DISCUSSION.

These experiments seem to be specially relevant to the problem of why a culicine mosquito, but not an anopheline, may act as a vector of an encephalitis virus after virus is fed. MVE virus has multiplied after injection into the body cavity both of culicine and anopheline mosquitoes. When one compares *C. annulirostris* and *A. annulipes* it is evident that virus injected parenterally showed a high titre in the gut of the culicine several days later, but not in the anopheline, pointing rather directly to multiplication in the enteral tissues of the culicine and not in the anopheline. This step appears to be necessary in order that virus may gain entry to the non-enteral tissues, including the salivary glands, where it undergoes further multiplication and thus makes possible the secretion of infective saliva when the mosquito bites a fresh host. We conclude that what determines whether or not an encephalitis virus is capable of undergoing a biological cycle in a given mosquito is the capacity of the cells of the gut to allow multiplication of the virus. Only when this has occurred can invasion of the other tissues of the mosquito take place.

The only natural vectors of encephalitis viruses appear to be culicine mosquitoes, whereas anopheline mosquitoes have not been incriminated as vectors.

St. Louis encephalitis (SLE) virus was not transmitted either by *A. punctipennis* or *A. maculipennis freeborni* (Hammon and Reeves, 1943), JBE virus was not transmitted by *A. maculipennis freeborni* (Reeves and Hammon, 1946). *A. quadrimaculatus* was not a vector of EEE or WEE virus (Merrill *et al.*, 1934); and MVE virus did not multiply after it was fed to *A. annulipes* (McLean, 1953a). Usually the amount of virus in the mosquito fell to zero by the second day after an infective blood meal, but Reeves and Hammon (1946) recorded the persistance of JBE virus in *A. maculipennis freeborni* as long as 8 days on one occasion, but the mosquito did not transmit virus by biting a chicken. Although the feeding habits and geographical distribution of *A. annulipes* (Reeves, *et al.*, 1954) would be consistent with the possibility that this mosquito may be a vector of MVE, the present work suggests that MVE virus is not carried by *A. annulipes* because anopheline gut is incapable of supporting the multiplication of virus.

A finding somewhat analogous to the multiplication of CF_5 virus after injection or feeding, plus gut puncture, or MVE virus multiplication in *A. annulipes* after injection was recorded by Merrill and Tenbroeck (1935), who found that certain strains of *Aedes aegypti* did not support the multiplication of Eastern equine encephalomyelitis (EEE) virus after virus was fed, but virus multiplication occurred if the gut of the mosquito was punctured immediately after an infective blood meal.

Viruses such as influenza and neurovaccinia did not multiply after they were fed to mosquitoes or after they were injected. This suggests that mosquito cells possess a high degree of specificity in regard to the type of virus whose multiplication they will support. It would appear that only viruses of the arthropod-borne group, which includes encephalitis viruses, are capable of multiplication in mosquitoes.

SUMMARY.

MVE virus multiplied in *C. annulirostris* and *Aedes queenslandis* after injection into the body cavity. Maximum amounts of virus were recovered on the second and subsequent days after injection, and virus was transmitted when mosquitoes bit chickens on the third and subsequent days.

MVE virus multiplied in *Anopheles annulipes* after injection, but not after virus was fed. Maximum amounts of virus were recovered on the sixth and subsequent days after injection.

CF_5 virus multiplied in culicine mosquitoes to a significant degree only after gut puncture or injection, maximum amounts being attained by the third day.

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REFERENCES.

Anderson, S. G., White, J. and McLean, D. M. (1954): Med. J. Austral., 1, p. 113.
Hammon, W. McD. and Reeves, W. C. (1943): J. exp. Med., 78, p. 241.
Hurlbut, H. S. (1951): Amer. J. trop. Med., 31, p. 448.
McLean, D. M. (1953a): Austral. J. exp. Biol., 31, p. 481.
McLean, D. M. (1953b): *Ibid.*, 31, p. 491.
Merrill, M. H., Lacaillade, C. W. and Tenbroeck, C. (1934): Science, 80, p. 251.
Merrill, M. H. and Tenbroeck, C. (1935): J. exp. Med., 62, p. 687.
Myers, K. (1954): Personal communication.
Reeves, W. C., French, E. L., Marks, E. N. and Kent, N. E. (1954): Amer. J. trop. Med. Hyg., 3, p. 147.
Reeves, W. C. and Hammon, W. McD. (1946): J. exp. Med., 83, p. 185.
Watson, B. K. (1952): *Ibid.*, 96, p. 653.

ASPARTIC ACID DEAMINATION BY INTACT PROTEUS X-19

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The anaerobic deamination of aspartic acid by agar-grown *Proteus X-19* is markedly stimulated by nucleosides, nucleotides and a number of fermentable compounds (Trudinger, 1951). Presumptive evidence was obtained that stimulation is a function of the fermentation of the compounds studied (except glutathione) and differs therefore from the activations studied by Gale (1938) and Lichstein and others (see review, Lichstein, 1951) which appear to be "coenzyme effects."

Stimulation in whole cells is partly due to a reversal of inhibition by sodium ions, found in media containing high concentrations of sodium (Trudinger, 1954). However, a number of substances increase the deamination rate in media containing non-inhibitory amounts of sodium. The present paper describes further experiments designed to elucidate the nature of this secondary stimulation.

Materials and Methods.

The bacterial strains and general methods used in this investigation have been described in earlier papers (Trudinger, 1951; 1953; 1954).

Additional estimations were as follows: glucose (Folin and Malmros, 1929); ribose (Meijbaum, 1939); lactate (Barker and Summerson, 1941); succinate (Krebs, 1935).

Gas exchanges were measured by the conventional Warburg technique (Umbreit *et al.*, 1949). Nitrogen was purified by passage over heated copper turnings followed by concentrated H_2SO_4, caustic soda and water. Yellow phosphorus was placed in the centre well of the Warburg cup to absorb any traces of oxygen. Hydrogen was purified by passage through chromous chloride, caustic soda and water. For hydrogen exchanges yellow phosphorus was placed in the centre well and caustic soda (0·2 ml. of 5N) in one side-arm.

Unless otherwise stated, reactions were performed at 38° C. in a final volume of 3 ml. Q values referred to µl. of material absorbed or evolved per mg. dry wt. per hour. Absorptions are indicated by a minus sign.

Cell-free aspartase.

Cell-free aspartase was prepared by mechanical disintegration with glass beads (Nossal, 1953). In general, 10 ml. of bacterial suspension (*ca.* 50 mg. dry wt.) in M/10 phosphate pH 7, were shaken at 0° C. for 1–2 minutes with 10 gm. No. 14 Ballotini glass beads. The presence of phosphate was essential to obtain active extracts. After centrifugation at 5,000 r.p.m. (*ca.* 3,500 *g.*) all activity was found in the supernatant. The cell-free enzyme exhibited the same kinetics and properties as the enzyme in toluene-treated cells (see Trudinger, 1953) except that it was less sensitive to inhibition by phosphate.

EXPERIMENTAL.

Stimulation by glucose.

Previous results (Trudinger, 1951) suggested that stimulation of the anaerobic deamination of aspartate by glucose was related directly to glucose fermentation, ceasing when fermentation was complete. On this basis, the rate of anaerobic removal should be at least 0·9 μmol glucose/mg. cells/hour whereas observed rates did not exceed 0·5 μmol glucose/hour. In many cases the rate of glucose removal *per se* was almost zero, yet the relationship between stimulation and concentration of glucose was very similar to that found when the anaerobic metabolism of the sugar was more rapid.

However, in the presence of aspartate the anaerobic removal was greatly increased (Table 1) and was sufficient to explain its relationship to stimulation.

TABLE 1

Anaerobic Fermentation of Glucose and Lactate.

(0·066M K: Na phosphate pH 7·2; 60 mins, anaerobic.)

Expt.	Cells (mg. dry wt.)	Substrate (μmol/3ml)	Additions 10^{-3}M	Substrate (μmol/3ml)
1	2·5	Glucose (2·9)	None	−0·10
			L-aspartate	−2·75
			Fumarate	−2·75
2	1·78	Glucose (2·00)	None	−0·65
			Fumarate	−1·93
			L-malate	−1·88
			Oxalacetate	−1·92
			Pyruvate	−1·93
3	1·86	Lactate (1·73)	None	−0·20
			L-aspartate	−0·99
			Fumarate	−1·02

The effect was exhibited equally by fumarate, malate, oxalacetate and pyruvate. The enhanced glucose removal during aspartate deamination therefore was probably due to fumarate produced and not related directly to the deamination reaction *per se*.

The effect of fumarate on glucose metabolism appeared to be similar to that described by Krebs (1937) who, on the basis of succinate and CO_2 production, showed that *Escherichia coli* oxidized anaerobically many materials such as glucose, lactate, etc., at the expense of reduction of fumarate (see below).

Aspartate or fumarate stimulated also the anaerobic removal of lactate by *Proteus* (Table 1, Exp. 3).

Fig. 1a shows the relationship between stimulation and glucose metabolism. Stimulation ceased soon after glucose disappeared from the reaction mixture. Similar results were obtained when lactate or maltose replaced glucose.

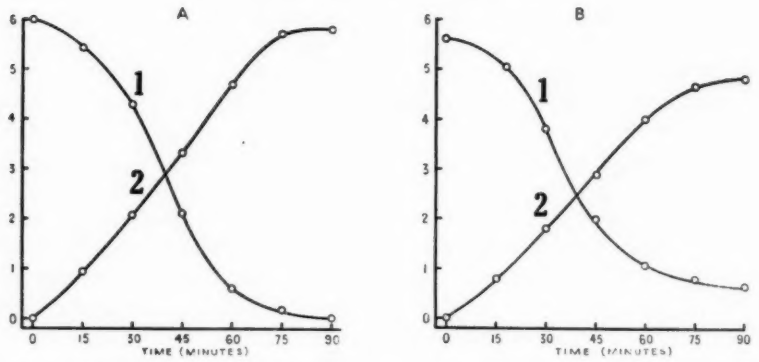

Fig. 1. *Glucose and ribose disappearance during deamination.* 0·01 M L-aspartate; 0·0033 M glucose or adenosine; Ca 0·9 mg. cells; 0·066 M K:Na phosphate pH 7·2; Anaerobic.

A. Curve 1. Glucose/3 ml.; 1 unit=30 μg.
Curve 2. Stimulation of deamination (total NH_3 minus basic NH_3 (3 ml.); 1 unit= 20μl NH_3.

B. Curve 1. Adenosine-ribose/3 ml.; 1 unit=25 μg.
Curve 2. Stimulation of deamination; 1 unit=20 μl. NH_3.

Stimulation by adenosine.

In the experience of the author identical or very similar results have been obtained whether glucose or adenosine was used to stimulate aspartase (Trudinger, 1951, 1953, 1954). On the other hand a specific rôle for adenosine (or adenylic acid) in the deamination reaction has been proposed by several workers (Gale, 1938; Lichstein and Christmas, 1948). Lack of activity by adenosine, ribose or mixtures of the two at first sight supports the latter view.

TABLE 2

Metabolism of Ribose and Adenosine.

(0·066M K: Na phosphate, pH 7·2; Incubation 60 mins.)

Expt.	Cells (mg. dry wt.)	Substrate (μmol/3ml)	Conditions	μmol ribose/3ml Final	Difference
1	1·54	Adenosine (4·65)	Aerobic	3·64	1·01
		Ribose (3·90)	Aerobic	3·80	0·10
2	1·54	Adenosine (4·65)	Anaerobic	3·88	0·77
		Ribose (3·90)	Anaerobic	3·90	0·00
		Adenosine (4·65) +0·01M L-aspartate	Anaerobic	2·92	1·73
		Ribose (3·90) +0·01M L-aspartate	Anaerobic	3·90	0·00
3	2·85	Adenosine (3·06)	Anaerobic	1·78	1·28
		Adenosine (3·06) +0·01M L-aspartate	Anaerobic	0·39	2·67
		Adenosine (3·06) +0·01M fumarate	Anaerobic	0·36	2·60

Stephenson and Trim (1938) demonstrated that *E. coli* fermented the ribose moiety of adenosine much more effectively than the free sugar. This has been ascribed by later workers (Cohen *et al.*, 1951) to a deficiency of the enzyme phosphorylating ribose, ribose-phosphate being formed during the splitting of adenosine.

With agar-grown *Proteus* adenosine-ribose was metabolised aerobically at about ten times the rate of free ribose. Anaerobically, the free sugar was not attacked to an appreciable extent under the conditions used (Table 2). The addition of aspartate or fumarate increased the anaerobic removal of adenosine ribose, and stimulation by the nucleoside ceased when the ribose residue was completely metabolised (Fig. 1b). Thus adenosine appeared to stimulate simply by supplying a utilisable form of ribose.

Proteus grown in heart infusion broth, particularly in the presence of ribose, metabolized free ribose anaerobically. Under these conditions the pentose stimulated anaerobic aspartate deamination to nearly the same extent as glucose and adenosine (Table 8).

Effect of cyclohexanol on deamination.

This compound was tested originally as a specific inhibitor of fumarase (Woolf, 1929; Ajl, 1950). However, fumarase in whole cells or in extracts was inhibited only at concentrations of cyclohexanol, which affected also many other enzymatic reactions. On the other hand, stimulation by glucose or adeno-

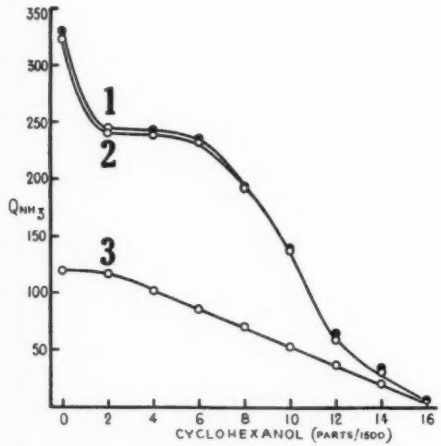

Fig. 2. *Cyclohexanol on anaerobic deamination.* 0·00375 M L-aspartate; 0·066 M K:Na phosphate pH 7·2; 0·46 mg. cells; Anaerobic.
Curve 1. 10^{-3} M adenosine added.
Curve 2. 10^{-3} M glucose added.
Curve 3. No additions.

sine was markedly reduced at concentrations of the inhibitor which did not affect significantly the basic deamination rate. Thus 1 to 2 parts per 1,500 cyclohexanol reduced stimulation by up to 40 p.c.: furthermore, a concentration of 6–8 parts per 1,500 was required to produce further inhibition (Fig. 2). At the higher concentrations the basic activity was also reduced. Complete inhibition of anaerobic deamination occurred at 15–16 parts per 1,500 cyclohexanol.

Low concentrations of cyclohexanol also reduced stimulation of anaerobic deamination by lactate, pyruvate and glutathione (Table 4). In the latter case stimulation was almost completely abolished. Stimulation by formate was unaffected.

Aerobically, the primary drop in activity at low inhibitor concentrations was not observed; the pattern of inhibition was essentially the same as the second phase of the curve found anaerobically in the presence of stimulators. Activity of toluene-treated cells was less susceptible to the inhibitor, about 30 p.c. of the initial activity remained in the presence of 20 parts per 1,500 cyclohexanol,

TABLE 3

Effect of Cyclohexanol on Aspartase Activity of Toluene-treated cells and Cell extracts.
(0·066M K:Na phosphate, pH 7·0; 0·01M $MgCl_2$ Incubation 60 mins., aerobically.)

Cyclohexanol parts per 1500	Toluene-treated Cells (QNH_3) (0·0025M L-aspartate)	Extract μlNH_3/3ml (0·01M L-aspartate)
0	131	201
6	127	—
10	115	197
12	104	—
14	90	—
16	75	—
20	50	190

TABLE 4

Effect of Cyclohexanol on the Stimulation by Lactate, Pyruvate, Glutathione and Formate.
(0·0025M L-aspartate; 0·066M K:Na phosphate, pH 7·0: Anaerobic.)

Expt.	Additions	QNH_3	
		No inhibitor	1/500 cyclohexanol
1	None	109	104
	Lactate (0·001M)	302	182
	Pyruvate (0·001M)	224	190
2	None	109	105
	Glutathione (0·001M)	166	109
3	None	110	107
	Formate (0·0033M)	180	175
	None	110	35[1]
	Formate (0·0033M)	180	58[1]

[1] 4/500 cyclohexanol.

both aerobically and anaerobically. Cell-free aspartase was almost unaffected by 20 parts per 1,500 cyclohexanol (Table 3).

The effect of cyclohexanol was not the same as of toluene, since Mg^{++} did not reactivate.

In an attempt to localize the action of the inhibitor, its effect on a number of other enzyme reactions was studied. Dehydrogenases (as measured by methylene blue reduction) were found to be extremely sensitive to low concentrations of the inhibitor. Reduction times with glucose, adenosine, lactate, pyruvate, maltose, glycerol, formate, glutathione, succinate, fumarate and malate were increased 2–7 times by the addition of 3 parts per 1,500 cyclohexanol. Quastel and Woolridge (1927) have observed also that a number of dehydrogenases were destroyed by treating *E. coli* with cyclohexanol.

The experiments described so far were carried out in a medium containing sufficient sodium to reduce the basic deamination rate by about 50 p.c. (Trudinger, 1954). In media containing non-inhibitory amounts of sodium, 1/500 cyclohexanol almost completely abolished stimulation of anaerobic deamination by glucose, adenosine, pyruvate and lactate, but had little or no effect on the basic activity (Table 5). Aerobic deamination was again unaffected.

TABLE 5

Effect of Cyclohexanol on Deamination in Low Sodium Medium.
(0·0025M L-aspartate; 0·066M K: K phosphate, pH 7·0; 0·02M NaCl Anaerobic.)

Additions (0·001M)	QNH_3	
	No inhibitor	1/500 cyclohexanol
None	219	204
Glucose	308	236
Adenosine	316	234
Pyruvate	280	218
Lactate	350	200

The decrease in stimulation by glucose, etc., on addition of small amounts of cyclohexanol, was the same in the presence or absence of inhibitory levels of Na^+. This indicated that cyclohexanol was inhibiting primarily the secondary stimulation and not reversal of sodium inhibition.

Effect of iodoacetate on deamination.

10^{-2} M iodoacetate completely inhibited the anaerobic deamination of aspartate both in the presence and absence of stimulators (Table 6). Stimulation by glucose or adenosine was more sensitive than the basic activity, being abolished by 10^{-4} M iodoacetate, which reduced the basic deamination by only 10–20 p.c. In the presence of 10^{-3}–10^{-4} M iodoacetate, the addition of glucose or adenosine

TABLE 6

Effect of Iodoacetate on the Deamination of Aspartic Acid.
(0·0025M L-aspartate; 0·066M K: Na phosphate, pH 7·2.)
All results expressed as QNH_3 except expt. 5, expressed as μlNH_3/3ml/hour.

Expt.	Conditions	Additions (0·001M)	Iodoacetate (M)			
			—	0·001	0·001	0·0001
1	Whole cells Anaerobic	None	128	0	81	111
		Glucose	266	0	64	84
		Adenosine	272	0	34	47
2	Whole cells Anaerobic	None	106	3	38	74
		Lactate	350	8	191	290
		Pyruvate	250	2	155	230
3	Whole cells Aerobic	None	280	5	253	275
4	Toluene-treated cells Anaerobic	$MgCl_2$ (0·01M)	135	75	127	
5	Cell extract Aerobic	$MgCl_2$ (0·01M)	202	150	200	201

actually reduced the deamination rate. The reason for this is not known. Iodoacetate appeared to uncouple fermentation from stimulation since 10^{-3}–10^{-4} M iodoacetate did not affect appreciably fermentation of glucose or adenosine. Its action differed from that of cyclohexanol since reversal of sodium inhibition was also inhibited by iodoacetate. The phenomenon was not observed with lactate or pyruvate, stimulation by which was less sensitive to the inhibitor than the basic deamination.

Aerobic deamination was completely inhibited by 10^{-2} M iodoacetate, but little affected by 10^{-3} M. Deamination by toluene-treated cells or cell extracts was unaffected by 10^{-3} M iodoacetate and inhibited only 25–40 p.c. by 10^{-2} M.

Effect of molecular hydrogen.

The results obtained with the use of inhibitors support the view suggested in earlier papers (Gale, 1938; Trudinger, 1951) that there is a relationship between reducing activity and stimulation of aspartate deamination. Furthermore, among those compounds stimulating deamination, the most efficient in this respect reduced methylene blue in the Thunberg the most rapidly (unpublished results).

This prompted a study of the deamination reaction under hydrogen. *Proteus* is known to be a powerful source of hydrogenase (Hoberman and Rittenburg, 1943) and the strain used in this study, when grown on agar, had an activity ($Q\text{-}H_2$) of about 150, using fumarate as a hydrogen acceptor.

With agar-grown cells hydrogen was more effective than glucose or adenosine in stimulating deamination of aspartate (Table 7). The addition of glucose

TABLE 7

Deamination of Aspartic Acid under Hydrogen.
(0·01M L-aspartate; 0·066M K: Na phosphate, pH 7·2.)

Additions (0·001M)	QNH_3		
	Nitrogen	Hydrogen	In vacuo
None	120	400	118
Glucose	240	398	
Adenosine	248	414	

or adenosine did not affect the deamination under hydrogen. The hydrogenase activity of cells grown aerobically in broth was reduced, and in this case stimulation by hydrogen was approximately the same as that by glucose or adenosine. Deamination under hydrogen was unaffected by high concentrations of sodium and proceeded at a linear rate to completion. Cyclohexanol inhibited in the same manner described for glucose. Thus hydrogen appeared to reproduce the effects of other stimulators studied.

Toluene-treated cells, prepared as described earlier (Trudinger, 1953), possessed hydrogenlyase activity, but hydrogen did not increase the rate of aspartate deamination except when the reaction was nearing equilibrium.

Hydrogen production could not account for stimulation by other compounds studied. Only formate gave significant amounts of hydrogen when incubated with whole cells. However, in contrast to stimulation by hydrogen, that by formate was not inhibited by low concentrations of cyclohexanol (Table 4). Also formate did not stimulate in low sodium media even though hydrogenlyase activity was unaffected. It would appear, therefore, that formate stimulated only by reversing sodium inhibition.

Effect of stimulators on fumarate metabolism.

In view of the marked inhibitory effect of fumarate on aspartase (Trudinger, 1953) it was necessary to determine whether stimulation in whole cells could be the result of increased fumarate removal.

Fumarate was fermented anaerobically by *Proteus* with the production of one molecule each of succinate and CO_2 for every 2 molecules of fumarate disappearing. It should be mentioned that this ratio is somewhat different to that observed by Krebs (1937) for *E. coli*, and represents an incomplete oxidation of fumarate. The nature of other residual products has not been determined, but no pyruvate, lactate, ethanol, acetaldehyde or acetoin were detected; some volatile acid was present.

Glucose, adenosine and pyruvate did not affect the removal of fumarate, but increased markedly the succinate produced. Glutathione had no effect on either

fumarate metabolized or succinate production. Lactate increased succinate formation and, to a lesser extent, increased fumarate removal. Formate and hydrogen increased fumarate removal and succinate formation to about the same extent.

Fumarate produced during aspartate deamination appeared to follow the same pathway as added fumarate. On the addition of glucose, adenosine, lactate, formate, pyruvate or hydrogen, the additional aspartate removed resulted in an equivalent increase in succinate. Only in the cases of hydrogen, lactate or formate was there any significant decrease in the fumarate concentration during deamination. In the presence of glutathione, both succinate and fumarate production increased; the ratio of succinate produced to fumarate removed was that observed in the non-stimulated reaction.

It would appear from these results that stimulation of deamination cannot be explained on the basis of modified fumarate metabolism. The results obtained with the glucose-grown cells, in which succinate production from fumarate is markedly decreased, support this view (see below).

The nature of the aerobic deamination.

Fumarate was oxidized by agar-grown *Proteus* at very rapid rates and only traces of dicarboxylic acid were detected in the medium during the aerobic deamination of aspartate. It has been suggested (Trudinger, 1951) that the high aerobic rates of deamination may be due to stimulation of the reaction by the subsequent oxidation of fumarate. It was first necessary to examine the possibility of an oxidative deamination of the amino-acid.

Such an investigation depends largely on indirect evidence since the same end products would arise whether oxidation of the aspartate occurred directly or via fumarate. It is significant that no enzyme has yet been described which deaminates L-aspartate via oxidation or dehydrogenation.

The following evidence suggests strongly that oxygen uptakes observed with aspartate as substrate are due to oxidation of the fumarate formed by deamination.

Cells grown under different conditions (variation in pH and temperature, in broth or on agar, aerobically or anaerobically, and with and without the addition of aspartate or fumarate) differed widely in their ability to oxidize aspartate. In all cases fumarate and aspartate were oxidized at approximately the same rates. Also mixtures of fumarate and aspartate were oxidized at about the same rate as the equivalent concentration of either.

Reduction of methylene blue in the Thunberg tube was a function of fumarate concentration, and with aspartate as substrate, was less rapid than with fumarate in equivalent concentration.

No immediate drop in oxygen consumption occurred when aerobic deamination of aspartate was complete.

Iodoacetate and cetyltrimethyl ammonium bromide, at concentrations which had no effect on deamination, reduced oxygen uptake by 50 p.c. or more and fumarate oxidation to the same extent.

Cyanide at 10^{-2} M inhibited oxidation 100 p.c. and reduced deamination to that obtained anaerobically in the absence of stimulators (Trudinger, 1951). By the use of graded amounts of cyanide it was found that oxygen uptake could be reduced by about 70 p.c. before aerobic deamination was affected even in cases where the aerobic deamination was only 40 p.c. greater than the anaerobic. Aerobic deamination in the presence of 10^{-2} M cyanide was stimulated by glucose and adenosine.

Deaminase activity of carbohydrate-grown cells.

Stephenson and Gale (1937) showed that *E. coli* grown in media containing glucose possessed about 10–20 p.c. of the deaminating ability towards glycine, alanine and glutamate of cells grown in the absence of carbohydrate. Similar results were obtained with serine deaminase (Gale and Stephenson, 1938) and aspartase (Gale, 1938). These workers suggested that glucose suppressed the formation of the deamination enzymes. On the other hand, Boyd and Lichstein (1951) believe that reduction of aspartate, threonine and serine deaminases in glucose-grown cells was the result of dissociation of a coenzyme from the enzyme complex.

TABLE 8

Deamination of Aspartate by Carbohydrate Grown Cells.

Basic Medium 2·5% heart infusion broth +0·5% yeast extract (Difco) +M/25 phosphate initial pH 7·0-7·2. Activity measured in 0·066M K:Na phosphate pH 7·2 with 0·01M L-aspartate ±additions, 0·005M.

Expt.	Alterations to medium	Additions during deamination	QNH_3	
			Anaerobic	Aerobic
1	None	None	137	315
		Glucose	373	318
		Adenosine	370	313
2	+1% glucose	None	41	46
		Glucose	355	303
		Adenosine	370	324
3	+1% ribose	None	65	94
		Glucose	561	466
		Adenosine	590	510
		Ribose	450	466
4	+1% galactose	None	62	135
		Glucose	387	350
		Galactose	319	352
5	+1% lactate	None	66	274
		Glucose	830	790
6	+1% adenosine	None	91	314
		Glucose	835	802

With *Proteus*, the addition of glucose to the medium produced two main effects (Table 8). First the basic anaerobic deamination was lowered, but not the activity in the presence of stimulators. Secondly, aerobic deamination was markedly reduced (to little more than the basic anaerobic rate) and responded to the addition of stimulators. The aerobic deamination by cells grown in the absence of glucose was unaffected by the addition of stimulators.

These effects were observed only in broth media and were not due to changes in pH or the production of anaerobiosis. The effect could be reproduced by ribose and galactose, but not by fructose, mannose or lactose. Lactate and adenosine appeared to act similarly, but had an additional stimulatory effect on the overall deamination rate. Boyd and Lichstein (1953) have shown that xylose, glycerol, citrate, inositol and maltose reproduce the effect of glucose.

If the contention of Boyd and Lichstein, that glycolysis resolves an enzyme-coenzyme complex, is correct, then incubation of the pre-formed enzyme with glucose should have the same effect as glucose present during growth. This has been tested in two ways.

1. Washed suspensions of *Proteus* grown on agar were incubated aerobically, or anaerobically, for 2–3 hours in phosphate at pH 7 with M/3 glucose. They were then centrifuged, washed and tested for aspartase activity.

2. Cells were grown for 12 hours at 37° C. in heart infusion broth without glucose. The suspension was then divided into three parts: (a) was harvested and tested for aspartase; (b) was incubated for a further 6 hours before harvesting; (c) was also incubated for 6 hours after the addition of 1 p.c. sterile glucose. In neither series of experiments did glucose depress the aerobic aspartase activity nor was there any stimulation when glucose was added during deamination. In the second series of experiments (2) glucose tended to enhance the deamination rate. In one experiment the respective QNH_3 values were: (a) 345, (b) 380, (c) 520.

During the 6 hours' incubation in the second series of experiments up to 240 p.c. more growth occurred (as gauged by increase in mg. dry wt.). These cells retained the aspartase characteristics of the initial generation of cells and were not affected in the same way as cells grown in the presence of glucose from inoculation. The possibility that "normal" cells stimulated the activity of "glucose" cells was tested by studying the activity of mixtures of normal and glucose-grown bacteria. The deamination rate was the sum of that obtained with either alone.

Oxidations by glucose-grown cells.

Epps and Gale (1942) demonstrated that some dehydrogenases were deficient in *E. coli* grown on glucose, while Back and Mitchell (1952) showed that succinate oxidation by glucose-grown *E. coli* was, at least in the early stages of the reaction, much slower than that by bacteria grown in the absence of glucose.

With *Proteus*, growth on glucose diminished markedly oxidation of aspartate, fumarate and malate (Table 9). Succinate, lactate, ethanol, formate and

TABLE 9

Oxidations by Glucose-grown cells.

Basic medium as in Table 8. Reactions run in 0·066M K: Na phosphate pH 7·2. Rates expressed as QO_2 for the first 60 minutes.

Substrate 0·01M	Cells	
	Basic medium	+1% glucose
L-aspartate	205	3
Fumarate	190	14
L-Malate	185	46
Glucose	76	91
Lactate	195	55
Succinate	208	30
Ethanol	53	25
Formate	155	15
Acetate	11	0

acetate oxidations were reduced to about the same degree. The initial oxidation rate of glucose was unaffected by growth on glucose, but in contrast with cells grown in the absence of glucose, ceased when oxygen consumption reached that expected for oxidation to the acetate stage, and volatile acid accumulated.

Reduced oxidation rates were observed also when cells were grown on ribose, adenosine or lactate, but not when grown with sugars which had no effect on aspartase activity.

Oxidation of aspartate and fumarate was restored when fumarate was included in the medium containing glucose. Concurrently, the aerobic aspartase activity was regenerated (Table 10). Similar results were obtained when aspar-

TABLE 10

Reversal of Glucose effect by Fumarate.

Basic medium and measurement of activities as in Tables 8 and 9.

Medium	Substrates	QNH_3		$Q\text{-}O_2$
		Anaerobic	Aerobic	
+1% glucose	L-aspartate	51	51	0
	L-aspartate+glucose	352	332	—
	Fumarate	—	—	8
+1% glucose	L-aspartate	87	449	167
+1% fumarate	L-aspartate+glucose	480	445	—
	Fumarate	—	—	140

tate or succinate replaced fumarate. Glucose was still rapidly metabolized when the dicarboxylic acids were present in the growth medium.

Fermentation of fumarate by glucose-grown cells.

With glucose-grown cells values of Q-fumarate of the order of 100 were obtained as compared with *ca* 250 with cells grown in the absence of glucose. The addition of glucose, lactate, adenosine, etc., to the system caused only slight increases in succinate production. Fumarate and malate accumulation was very marked during aspartate deamination by glucose-grown cells. Over 50 p.c. of the aspartate deaminated was recovered as fumarate plus malate, even in the presence of stimulators.

Effect of growth in glucose on aspartase activity of toluene-treated cells and extracts.

Comparison of aspartase-activity in toluene-treated cells and cell-free extracts indicated that preparations from glucose-grown cells possessed about 50 p.c. of the activity of those from cells grown in the absence of carbohydrate. It would be premature to say that the enzyme itself was decreased in concentration since the nature of any coenzymes involved is not fully elucidated. Biotin, adenylic acid, adenosine, yeast extract or extracts of non-glucose-grown cells did not increase the activity of preparations from glucose-grown cells.

Fumarase activity was also markedly lower in preparations from glucose-grown cells.

Boyd and Lichstein (1951) found that addition of high concentrations of adenylic acid, biotin or yeast extract to media containing glucose tended to offset the inhibitory effect of the sugar. In this laboratory no effect was found with biotin while the action of yeast extract could be explained by its dicarboxylic acid content. It is possible also, that adenylic acid may act like adenosine in enhancing overall aspartase activity while not affecting the relative effects of glucose.

Effect of substrate concentration on aspartase activity of whole cells.

It was shown (Trudinger, 1953) that although toluene-treatment of *Proteus* completely abolished stimulation, the activity of treated cells was much greater at high substrate levels than the aerobic deamination by whole cells. This study has been extended to include deamination under anaerobic conditions. The results are given in Fig. 3.

With toluene-treated cells the relationship between substrate concentration and deamination rate obeyed the equation of Michaelis and Menten (1913). With whole cells the reaction rate was little affected by increasing aspartate concentration between M/2000 and M/200 (using *ca.* 0·5 mg. cells); thereafter the activity increased with rising substrate concentration, but not to the extent found with toluene-treated cells. Below M/2000, deamination was almost complete in 15 minutes.

The existence of a plateau at low substrate levels suggested that at these concentrations aspartate was not entering the cell by simple diffusion but by

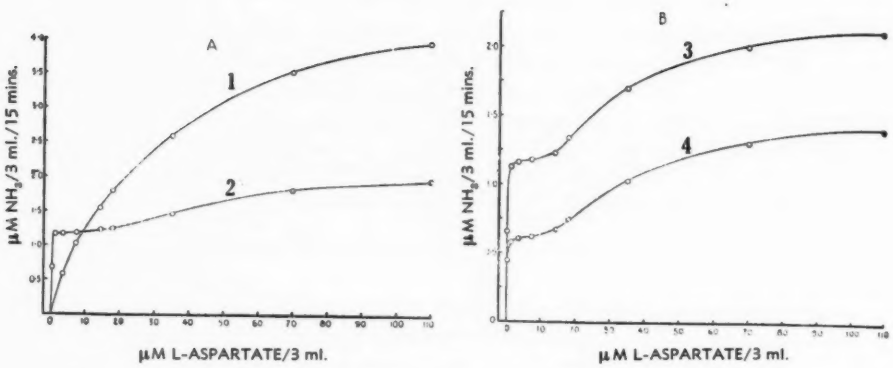

Fig. 3. *Effect of substrate concentration on deamination by whole cells.* 0·066 M K:Na phosphate pH 7·2 ca. 0·5 mg. cells.

Part A. Aerobic.
Curve 1. Toluene-treated cells $+10^{-2}$ M $MgCl_2$.
Curve 2. Whole cells. No additions.

Part B. Anaerobic.
Curve 3. Whole cells$+10^{-2}$ M glucose.
Curve 4. Whole cells. No additions.

some active process. At higher levels the external concentration may have been sufficient to "push" small amounts through the membrane over and above its "metabolic" entry.

Support for this concept lies in the partial reversal of iodoacetate inhibition by high concentrations of aspartate which, it will be recalled, has little effect on cell-free aspartase at concentrations below 10^{-2} M (Table 11). It is signi-

TABLE 11

Effect of Iodoacetate on Deamination at two Substrate Levels.
(0·066M K: Na phosphate, pH 7·2; 0·48mg cells; anaerobic.)

Iodoacetate Concentration	L-aspartate (0·0025M)		L-aspartate (0·025M)	
	QNH_3	% Inhibition	QNH_3	% Inhibition
—	130		202	
0·01M	0	100	50	83
0·002M	58	56	114	43

ficant that the differences between the deamination rates at 0·0025 M and 0·025 M aspartate in the presence and absence of inhibitor, were approximately the same, suggesting that iodoacetate inhibited the metabolic entry of aspartate but not the secondary mechanical entry.

The addition of stimulators during anaerobic deamination had little effect on the shape of the curve but raised markedly the "plateau." A similar but less marked effect was obtained by conducting the deamination in air. This suggested that the function of stimulating agents was to increase the efficiency of the metabolic uptake of aspartate.

While at low substrate concentration aspartate deamination was lower in toluene-treated cells than in whole cells, at high levels of aspartate the activity of treated cells was up to twice that found with stimulated whole cell deamination. Since the activity of toluene-treated cells was measured under suboptimal conditions (e.g., an inhibitory phosphate concentration was present to stabilize the enzyme—Trudinger, 1953), it is obvious that the activity of toluene-treated cells was sufficient to explain whole cell activity if some internal concentration of aspartate occurred. Such would be the result if aspartate was taken up by an "active" process.

It is interesting in this respect that the effect of glucose on acetoin formation from pyruvate has been attributed to a stimulation of pyruvate uptake (Moat and Lichstein, 1953).

DISCUSSION.

It has been shown previously (Trudinger, 1954) that in media containing high concentrations of sodium, stimulating agents reverse the inhibition of aspartate deamination by sodium ions. The results reported in this paper suggest that the additional stimulation, found on addition of glucose, etc., to media containing non-inhibitory amounts of sodium, is the result of an increased uptake of aspartate into the cell. It is obvious from a comparison with the activity in toluene-treated cells (in which permeability barriers are markedly reduced) that the enzyme in whole cells does not become saturated with substrate so that deamination rates will be a function of the rate of aspartate entry into the cell.

Most of the compounds studied exhibit both effects to varying extents. Exceptions are formate, which only reverses sodium inhibition, and glutathione, which is active only in increasing aspartate penetration.

The mechanism by which uptake of the amino-acid is effected is not clear. In the assimilation of glutamate by Gram-positive bacteria and yeast, glucose appears to function as an energy source (Gale, 1947; Taylor, 1947). However, in the present case stimulation by hydrogen and glutathione and the lack of inhibition by 2:4 dinitrophenol (cf. Gale, 1951; Gale and Paine, 1951) appear to make such a theory untenable.

There is some suggestion that stimulation of anaerobic deamination may be a function of the reducing ability of the stimulating agents. It is of interest that a relationship between permeability and the maintenance of free —SH groups

has been suggested by several workers (Jacobs and Corson, 1934; Davson, 1943; Barron, 1951). On the other hand the almost immediate cessation of stimulation after fermentation of glucose, adenosine, lactate, etc., is complete, is difficult to reconcile with such a theory.

It may well be that the mechanism of aspartate assimilation varies with the system used. Such differences are suggested by the following facts.

(a) Cyclohexanol at low concentrations appears to inhibit aspartate assimilation under the influence of glucose, adenosine, hydrogen, lactate and glutathione. The same concentrations have no effect on assimilation under the influence of the oxidation or fermentation of fumarate.

(b) Iodoacetate at 10^{-3} to 10^{-4} M appears to uncouple fermentation of glucose or adenosine from assimilation, but has no marked effect on the basic anaerobic activity, aerobic deamination or stimulation by lactate or pyruvate.

With some bacteria, particularly strict anaerobes and strict aerobes, the formation of fumarate and malate cannot be demonstrated during aspartate deamination, but succinate accumulates (Cook and Woolf, 1928). In these cases toluene abolishes deamination completely. The suggestion was made, therefore, that here aspartate is deaminated by a reductive process. With *Proteus* grown in the absence of carbohydrate, the production of succinate during deamination in the presence of stimulators would agree with a reductive deamination in their presence. However, apart from a lowered basic activity, anaerobic deamination by glucose-grown cells appears to be similar to that by non-glucose-grown cells, but accumulation of succinate is markedly lowered. A reductive deamination is therefore not likely to occur.

While the present study has revealed no evidence for a coenzyme in the aspartase reaction, and adenosine seems to act simply by virtue of its fermentation, it should be stressed that the system is essentially different from that used by other workers (see Lichstein, 1951, for reference). The effects described in this and preceding papers are characteristic of fresh untreated cells, whereas the "coenzyme" effects of adenylic acid, biotin, etc., were obtained with cells treated to reduce their initial activities considerably. Furthermore, stimulations by adenosine or adenylic acid in cell-free extracts have been described (Gale, 1938; Lichstein, 1949).

That the superior deamination rates observed aerobically are the result of the oxidation of fumarate is indicated by the following:

(a) The concurrent reduction of fumarate oxidation and aerobic deamination of aspartate by (i) omission of K^+ or Na^+ (Trudinger, 1954); (ii) growth of the cells in glucose, or (iii) the addition of cyanide.

(b) Lack of evidence for an oxidative deamination of aspartate.

(c) Regeneration of fumarate oxidation and aspartate deamination on the inclusion of fumarate or aspartate in media containing glucose.

Growth in glucose appears to affect aspartase activity of the whole cell

indirectly by reducing fumarate metabolism. Some decrease in the activity of toluene-treated cells and extracts also occurs, but sufficient activity remains to account for the deamination rates observed with whole cells.

SUMMARY.

Stimulation of the anaerobic deamination of aspartic acids by sugars, nucleosides and materials such as lactate was a function of the fermentation of these compounds.

Iodoacetate and cyclohexanol inhibited deamination by whole cells, particularly stimulation, at concentrations which had little or no effect on aspartase in toluene-treated cells or extracts.

Molecular hydrogen was a powerful stimulator of anaerobic deamination by whole cells.

The rate of deamination in absence of stimulators appeared to be dependent on the metabolism of fumarate. The superior aerobic rates were correlated with oxidation of fumarate.

Addition of glucose to the growth medium appeared to reduce deamination of aspartate by decreasing fumarate metabolism.

Evidence is presented that one function of stimulating compounds was to increase the uptake of aspartate into the cell.

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REFERENCES.

Ajl, S. (1950): J. Bact., 59, p. 499.
Back, K. J. C. and Mitchell, R. (1952): Austral. J. sci. Res. Ser. B., 5, p. 256.
Barker, J. B. and Summerson, W. H. (1941): J. biol. Chem., 138, p. 535.
Barron, E. S. G. (1951): Advances in Enzymology, 11, p. 201.
Boyd, W. L. and Lichstein, H. C. (1951): J. Bact., 62, p. 711.
Boyd, W. L. and Lichstein, H. C. (1953): Proc. Soc. exp. Biol. Med., 82, p. 45.
Cohen, S. S., Scott, D. B. M. and Lanning, M. (1951): Fed. Proc. 10, p. 173.
Cook, R. P. and Woolf, B. (1928): Biochem. J., 22, p. 474.
Davson, H. (1943): Permeability of Natural Membranes, Cambridge and New York, p. 254.
Epps, H. M. R. and Gale, E. F. (1942): Biochem. J., 36, p. 619.
Folin, O. and Malmros, H. (1929): J. biol. Chem., 83, p. 115.
Gale, E. F. (1938): Biochem. J., 32, p. 1583.
Gale, E. F. (1947): J. gen. Microbiol, 1, p. 53.
Gale, E. F. (1951): Biochem. J., 48, p. 286.
Gale, E. F. and Paine, T. F. (1951): *Ibid.*, 48, p. 298.
Gale, E. F. and Stephenson, M. (1938): *Ibid.*, 32, p. 392.
Hoberman, H. D. and Rittenburg, D. (1943): J. biol. chem., 147, p. 211.
Jacobs, N. H. and Corson, S. A. (1934): Biol. Bull., 67, p. 325.
Krebs, H. A. (1937): Biochem. J., 31, p. 2095.
Lichstein, H. C. (1951): Vitamins and Hormones, IX, p. 27.

Lichstein, H. C. (1949): J. biol. Chem., 177, p. 125.
Lichstein, H. C. and Christmas, J. F. (1949): *Ibid.*, 175, p. 649.
Meijbaum, W. (1939): Z. physiol. Chem., 258, p. 117.
Michaelis, L. and Menten, M. L. (1913): Biochem. Z., 49, p. 333.
Moat, A. G. and Lichstein, H. C. (1953): J. Bact., 66, p. 324.
Nossal, P. M. (1953): Austral. J. exp. Biol., 31, p. 583.
Quastel, J. H. and Woolridge, W. R. (1927): Biochem. J., 21, p. 148.
Stephenson, M. and Gale, E. F. (1937): *Ibid.*, 31, p. 1316.
Stephenson, M. and Trim, A. R. (1938): *Ibid.*, 32, p. 1740.
Taylor, E. S. (1947): J. gen. Microbiol., 1, p. 86.
Trudinger, P. A. (1951): Austral. J. exp. Biol., 29, p. 117.
Trudinger, P. A. (1953): *Ibid.*, 31, p. 319.
Trudinger, P. A. (1954): *Ibid.*, in press.
Umbreit, W. W., Burris, R. H. and Stauffer, J. F. (1949): Manometric Techniques and Tissue Metabolism, revised ed., Burgess Publishing Co., Minneapolis, Minn.
Woolf, B. (1929): Biochem. J., 23, p. 472.

STUDIES IN CARBON TETRACHLORIDE POISONING

III. THE POSSIBLE RÔLE OF SULPHUR AMINO ACIDS IN DETOXICATION

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The addition of sulphur amino acids to a diet low in protein content will reduce the incidence of hepatic and renal injury caused by halogenated organic compounds (György, Seifter, Tomarelli and Goldblatt, 1946; Miller and Whipple, 1942; Koch-Weser, de la Huerga, Yesinick and Popper, 1953). An increased demand for these amino acids subsequent to chloroform poisoning is suggested by the finding of a decreased concentration of glutathione in the liver after oral administration of chloroform in oil (Binet, Weller and Goudard, 1937). A disappearance of non-protein sulphydryl (—SH) groups has also been observed when liver extracts were incubated with various chlorinated compounds (Bray, Thorpe and Vallance, 1952).

The aim of the present work was to try to investigate the possible significance of sulphur amino acids in the detoxication of carbon tetrachloride. Rats on diets either adequate or deficient with respect to sulphur amino acids were subjected to varying degrees of carbon tetrachloride poisoning and changes in the liver glutathione were determined. Additional experiments were performed with liver extracts. To these extracts were added carbon tetrachloride and other chlorinated compounds, and the disappearance of non-protein —SH groups was estimated.

Materials and Methods.

Animals.

Male albino rats were used for all experiments. Group I consisted of adult rats, groups II and III were young rats, 10 weeks old. Groups I and II were fed the stock-breeding diet containing 22 p.c. protein and 0·9 p.c. sulphur amino acids. At weaning, animals in group III were put on the sulphur amino acid deficient diet of Lindan and Work (1953). This diet contained 7 p.c. protein and only 0·3 p.c. sulphur amino acids.

Unless otherwise stated, the dose of carbon tetrachloride administered was 0·1 ml./100 gm. body weight. This was dissolved in an equal volume of liquid paraffin and injected intraperitoneally. The animals were not fasted and were killed with coal gas 24 hours later.

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This time interval was considered to be optimal as judged by changes in creatine metabolism (Eden, Harrison and Linnane, 1954).

Chemical methods.

In a glass homogenizer, 0·5 gm. of liver was ground with 5 ml. of cold water. The homogenizer and pestle were rinsed with 2 ml. of water. The proteins were precipitated with 1 ml. of 20 p.c. sulphosalicylic acid. The extract was centrifuged, filtered and diluted to 10 ml. with water.

Total —SH groups were estimated iodometrically (Leaf and Neuberger, 1947), glutathione by the nitroprusside test (Grunert and Phillips, 1951) and cysteine by the colour reaction with brucine (Nakamura and Binkley, 1948). Ascorbic acid was titrated with 2, 6-dichlorphenol indophenol (Leaf and Neuberger, 1947) and total nitrogen was determined by Nesslerization using the hydrogen peroxide digestion method of McKenzie and Wallace (1954).

Liver extracts for *in vitro* tests were prepared in the manner described by Bray *et al.* (1952). Blood was haemolysed with an equal volume of digitonin solution (20 μg./ml.) and buffer and test materials were added as for liver extracts.

RESULTS.

In vivo *experiments.*

All results were expressed as mg. per 100 gm. body weight (see Table 1) because in many cases of liver damage there is a change in gross liver composition. This method is following the convention used by Lindan and Work (1953).

In all three groups, 24 hours after injecting carbon tetrachloride, there was a significant increase of glutathione (for groups I and II $P<0·05$, for group III $P<0·01$). Liver weights and total nitrogen were also increased, but the increase

TABLE 1

Effect of carbon tetrachloride on liver composition.

	No. of Animals	Body wt. (gm.)	Liver wt. (gm/100 gm body wt.)	Liver glutathione (mg./100 gm. body wt.)	Liver Nitrogen (mg./100 gm. body wt.)
Group I					
Adults, stock diet					
control	12	277±10·25*	3·63±0·08*	8·48±0·42*	111±5·7*
injected	12	296±8·85	3·95±0·10**	9·99±0·56**	119±6·9
Group II					
Young, stock diet					
control	8	126±6·08	4·82±0·12	6·87±0·57	134±7·0
injected	8	111±4·84	5·15±0·32	15·9±3·61**	152±9·9
Group III					
Young, sulphur amino acid deficient diet.					
control	8	73±3·94	5·09±0·22	3·48±0·31	—
injected	8	66±3·68	5·72±0·34	5·27±0·40***	—

* Mean ± standard error of mean.
** Mean significantly different from control group at 5 p.c. level.
*** Mean significantly different from control group at 1 p.c. level.
} Using Students t test.

in nitrogen was not significant. No changes were observed in the blood glutathione level.

Four young animals were injected with twice the usual dose of carbon tetrachloride. In these animals, the glutathione level was even higher than in those which received only 0·1 ml./100 gm. body weight (18·1 and 15·9 mg. glutathione/100 gm. body weight respectively). The effect of repeated injections was also tried. Three adult animals were injected on three consecutive days with 0·1 ml./100 gm. body weight and were killed 24 hours after the last injection. Again the glutathione level was higher than in the rats injected once only (12·2 and 9·99 mg. glutathione/100 gm. body weight respectively).

In many conditions changes in glutathione are paralleled by changes in ascorbic acid, hence liver ascorbic acid levels were also determined, but no changes were observed.

In vitro *experiments.*

Figures in Table 2 show some of the typical results of experiments with extracts of normal liver. These figures were all obtained by iodine titration as in similar experiments of Bray *et al.* (1952).

TABLE 2

The effect of chlorinated compounds on the stability of —SH groups.
Each incubation mixture contained 130 mg./ml. homogenised liver in 0·1 M phosphate buffer at pH 7·8. The homogenates were reinforced with cysteine to give an initial concentration of -SH groups of 22 mM. They were incubated in air at 37° C without shaking.

	No. of Expts.	—SH present after 4 hrs. (mM)	—SH lost (mM)
Control	5	16·02±1·22*	5·22±0·82*
Chloral hydrate (24 mM)	5	6·54±0·83	14·72±1·37**
Carbon tetrachloride (24 mM)	5	16·60±1·93	4·78±0·81

* Mean ± standard error of the mean.
** Mean significantly different from control group at 1 p.c. level, using Students t test.

When the extracts were incubated with cysteine alone, about 20 p.c. of the —SH groups disappeared in 4 hours. In the presence of chloral hydrate three times as much cysteine disappeared, thus confirming the results of Bray *et al.* (1952). On the other hand, the loss of cysteine in the presence of carbon tetrachloride was the same as that in the control experiments. The disappearance of cysteine was also followed by the more specific brucine methods, in which case all values were about 5 p.c. lower, but the effects of chlorinated compounds were the same. Chloroform, like carbon tetrachloride, had no effect on the disappearance of —SH groups. Similar results were also obtained when cysteine was replaced by glutathione, and the disappearance of glutathione was followed iodometrically and by the nitroprusside method.

In the experiments reported in Table 2 the extracts were incubated in air without shaking. This was done in order to reduce the non-specific oxidation of —SH groups by oxygen. Similar results to those shown in Table 2 were obtained when the samples were shaken under nitrogen.

In carbon tetrachloride poisoning the red cells are also damaged (Case, 1946). Therefore several experiments were set up to see whether this poison hastens the disappearance of the free —SH groups of red blood cells; again no difference was observed between the blank and the test extracts.

DISCUSSION.

Whether carbon tetrachloride was injected once or repeatedly, no reduction in liver glutathione level was observed. Thus the claim of Binet *et al.* (1937) that chlorinated hydrocarbons reduce the liver glutathione could not be confirmed. These authors used the non-specific iodometric method for the determination of glutathione.

In vitro studies also gave no indication of a combination of glutathione with carbon tetrachloride. A reaction occurs readily between —SH groups and halogens that are attached to the carbon atom adjacent to a carbonyl group (Dixon and Needham, 1946). With this type of compound the expected decrease of —SH groups in liver extracts was found, but no such decrease could be obtained in the case of the chlorinated hydrocarbons.

On the other hand, the increased levels of glutathione observed may be related to the active liver regeneration which occurs subsequent to necrosis (Campbell and Kosterlitz, 1948; Koch-Weser, Farber and Popper, 1951). Some of the findings obtained in the present investigation suggest that 24 hours after injection regeneration rather than necrosis was the important process occurring in the liver. The total liver nitrogen increased to approximately the same degree as did the liver weights, whereas in necrosis the increased liver weights are caused mainly by an increase in water content (Lindan and Work, 1953). The rise of glutathione in the liver was even larger than that of total nitrogen. Increased amounts of glutathione in the liver during regeneration following hepatectomy have been previously reported (Christensen, Rothwell, Sears and Streicher, 1948).

The magnitude of glutathione increase was affected by dietary treatment. This increase was much larger in group II, fed on adequate protein diet, than in group III, where the intake of sulphur amino acids was deficient. But even in this group an increased glutathione production occurred, and the sulphur amino acids presumably were obtained from tissue catabolism. These animals, like those in group II, lost about 10 p.c. of their body weight in 24 hours.

Although the present work gives no support for a direct detoxicating action of sulphur amino acids in carbon tetrachloride poisoning, these results are in

agreement with those obtained by György *et al.* (1946) and Miller and Whipple (1952). As already stated, these workers found that in animals kept on a protein deficient diet, sulphur amino acids helped to alleviate liver injury caused by halogenated hydrocarbons. As these sulphur amino acids are required for glutathione and protein synthesis, an adequate supply of these amino acids would be necessary for regeneration.

SUMMARY.

Young and adult rats were injected intraperitoneally with a single dose of carbon tetrachloride. Some of the young rats were previously kept on a sulphur amino acid deficient diet. Twenty-four hours after injection, the liver glutathione concentration had increased in all three groups. This increase was the largest in the young rats on an adequate diet, where the glutathione concentration was more than doubled.

Liver extracts incubated with cysteine or glutathione lost about 20 p.c. of sulphydryl groups in 4 hours. In the presence of chloral hydrate this was increased to over 60 p.c. On the other hand, the loss in the presence of carbon tetrachloride was the same as for the controls.

Thus the changes observed in the concentration of glutathione in the liver 24 hours after carbon tetrachloride poisoning are more likely to be associated with regeneration than necrosis, and so far no direct evidence has been obtained for an *in vivo* detoxication of carbon tetrachloride by glutathione.

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REFERENCES.

Binet, L., Weller, G. and Goudard, H. (1937): C.R. Soc. biol., 124, p. 1141.
Bray, H. G., Thorpe, W. V. and Vallance, D. K. (1952): Biochem. J., 51, p. 193.
Campbell, R. M. and Kosterlitz, H. W. (1948): Brit. J. exp. Path., 29, p. 149.
Case, R. A. M. (1946): Proc. Roy. Soc. B., 133, p. 235.
Christensen, H. N., Rothwell, J. T., Sears, R. A. and Streicher, J. A. (1948): J. biol. Chem., 175, p. 101.
Dixon, M. and Needham, D. M. (1946): Nature, 158, p. 432.
Eden, E., Harrison, D. D. and Linnane, A. W. (1954): Austral. J. exp. Biol., 32, p. 333.
Grunert, R. R. and Phillips, P. H. (1951): Arch. Biochem., 30, p. 217.
György, P., Seifter, J., Tomarelli, R. M. and Goldblatt, H. (1946): J. exp. Med., 83, p. 449.
Koch-Weser, D., de la Huerga, J., Yesinick, C. and Popper, H. (1953): Metabolism, 2, p. 248.
Koch-Weser, D., Farber, E. and Popper, H. (1951): Arch. Path., 51, p. 498.
Leaf, G. and Neuberger, A. (1947): Biochem. J., 41, p. 281.
Lindan, O. and Work, Elizabeth (1953): *Ibid.*, 55, p. 554.
McKenzie, H. A. and Wallace, H. S. (1954): Austral. J. Chem., 7, p. 55.
Miller, L. L. and Whipple, G. H. (1942): J. exp. Med., 76, p. 421.
Nakamura, K. and Binkley, F. (1948): J. biol. Chem., 173, p. 407.

THE LYSIS OF LEPTOSPIRES BY ANTISERUM

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The usual method of reading leptospiral agglutination tests is by direct examination under the darkground microscope of drops taken from the mixture of serum and suspension. Such a procedure encourages a study of the details of the action of immune serum on the leptospires and a number of workers have published descriptions of the accompanying phenomena. Many of their observations have been summarized by Uhlenhuth and Fromme (1930), and more recently by Rimpau (1943), who has himself carefully described the course of the reaction.

In general, the picture seen is as follows. The leptospires in the lower dilutions stick together to form clumps which consist of a loose network, while in the higher dilutions, although the number of leptospires is greatly diminished in comparison with the control, such loose floccular clumps are never found: the leptospires are said to have lysed. However, all traces of the leptospires do not necessarily disappear; more usually, besides a few unaltered free-swimming leptospires, granules of various sizes and shapes may still be seen under the darkground microscope, but these are regarded as the products of lytic degeneration. Schnüffner and Mochtar (1927), who made a special study of this process, described two different pictures; either all the leptospires disappear except for a few individual survivors, or else the granular disintegrating leptospires clump together to form "lysis balls"—bodies of various shapes and sizes which shine in a dazzling fashion in the dark field and have a smooth edge save for, perhaps, a few adhering spirochaetes. They also pointed out that this lysis was independent of the presence of complement as the same appearances were seen when the antiserum used had been previously inactivated. In this respect, of course, the lysis of leptospires differs strikingly from the lysis of other bacteria.

At times no agglutination is seen even in the lowest dilution of antiserum tested (usually 1/10): all the dilutions right out to the titre of the serum lyse the leptospires to varying degrees. Wolff (1925) has suggested that this might be explained by the lysin acting so quickly that the agglutinin has no time to act. The more usual picture of agglutination in the lower dilutions of antiserum accompanied by lysis in the higher shows some analogies to the Neisser-Wechsberg phenomenon, and indeed Rimpau, who considers the lysin a sub-

stance distinct from the agglutinin, explains it as due to the presence in the antiserum of a low titre of agglutinins together with a high titre of lysins which show the true Neisser-Wechsberg phenomenon. Schnüffner and Mochtar have suggested that a certain amount of dilution may be necessary to liberate the potential lytic energy of a specific substance, perhaps by dissociation of its molecules.

All workers are agreed that if the leptospires to be used in the agglutination test are first killed with formalin, lysis does not take place.

The object of this work was to investigate this unique bacteriolysis, and, in particular, to define the conditions under which lysis on the one hand, and agglutination on the other, occurred. However, it soon became apparent that the most difficult problem was to demonstrate unequivocal lysis and most effort was directed towards finding some suitable method.

Materials and Methods.

In most of the experiments the test organism was *Leptospira pomona*, but some of the results were checked using other serotypes including *L. icterohaemorrhagiae*, *L. australis A*, *L. pyrogenes*, *L. hyos* (syn. *L. mitis* Johnson) and *L. grippotyphosa*. Antisera were prepared in rabbits by the intravenous injection of cultures, and some limited use was made of the serum from naturally infected animals. Agglutination tests were carried out, unless otherwise indicated, using the method described by Schnüffner and Bohlander (1939) in which the final dilutions of the antiserum are 1/10, 1/30, 1/100, 1/300, 1/1000, 1/3000, 1/10000 and 1/30000.

The leptospires were grown in Vervoort's and Wolff's medium (van Thiel, 1948): a buffered dilute peptone water of pH 7·2, to which 10 p.c. of inactivated rabbit serum is added. By Vervoort's base is meant this medium without the addition of serum. The cultures were incubated at 30° C. for 4–7 days and usually contained about $100–200 \times 10^6$ organisms per ml.

EXPERIMENTAL.

As has been previously indicated, several workers have carefully followed the agglutination and lysis of leptospires under the microscope, but there is still some disagreement as to whether agglutination and lysis are separate phenomena, for instance, whether agglutination always precedes lysis. The progress of the reaction may be conveniently followed by mixing a drop of antiserum suitably diluted so that it should cause lysis (the precise dilution is found by a preliminary tube test) with a drop of suspension of living leptospires on a slide and observing the result under the darkground microscope using an oil-immersion objective (total magnification about 600×). The initial stages take place very rapidly even under a sealed coverslip and the general picture seems to be always the same: agglutination always precedes the formation of "lysis balls" or "degenerative granules." Individual leptospires sometimes form loops or rings, but more usually two, three or even more leptospires become at-

tached to one another and form a small clump with the constituent leptospires in very active movement, wriggling and twisting on one another. This leads to the formation of a small granule from which one or more leptospires, still actively moving, project. Usually, when these small clumps collide with one another, the projecting leptospires become intertwined and finally a larger granule is formed. These granules look exactly like the "lysis balls" formed when the antiserum reacts with leptospires in a test-tube, but they never become as big and they retain a wreath of projecting leptospires. However, the leptospires in slide preparations usually die when incubation is prolonged and the smaller size of the granules may be simply due to the fact that the leptospires make fewer contacts when confined within the almost two dimensional space between coverslip and slide. Also, leptospires may sometimes be seen projecting from the typical "lysis balls" which, in any case, are so large and refractile that a blinding light shines about them and makes it almost impossible to be sure whether radiating leptospires are present or not.

If the reaction is followed in drops which are left uncovered as in the agglutination test described by Krüger (1953), only the low power objective can be used. The picture seen is much the same as with coverslip preparations, but, after incubation in a moist chamber, the lysis granules are larger and it is often difficult or impossible to see leptospires projecting from their edges; in fact the granules merge by imperceptible steps into the "lysis balls" found when the reaction takes place in test-tubes. In the highest dilutions of an antiserum when the limit of its titre is being approached, the lysis granules often tend to be small in both tube and slide tests, and to show a number of radiating leptospires. This appearance is probably identical with that described as "agglutination terminale" by Bessermans, Wittebolle and Borchgrave (1938).

The size of the "lysis granules" or "balls" seems to be also dependent on the concentration of the leptospires. When the density of the leptospiral suspension was varied as well as the concentration of antiserum, only small granules were found in the more dilute suspensions in contrast to the usual large "lysis balls" seen in the suspensions of normal density.

It may be mentioned here that there seemed to be some relationship between the average length of the leptospires and the occurrence of floccular clumps rather than "lysis balls" in the lower dilutions of antiserum. Strains in which the leptospires were shorter than usual (say about 5μ in length) tended to form "lysis balls" even in the lowest dilutions.

It must be emphasized that individual leptospires were never lysed directly. Always they were agglutinated first and then formed the "lysis granules" or "lysis balls." The impression was formed that lysis did not take place at all—that all the leptospires that had disappeared from the suspension were now tightly wound together in the "lysis balls." Also the leptospires in these balls did not appear to be degenerate as, when typical "lysis balls" were teased out

with needles on a slide, numerous actively moving leptospires could always be seen where the ball had been torn.

One possible way of determining whether leptospires are, or are not, lysed seemed to be to immobilise them in some viscous or solid medium while the antiserum acted on them. The substances tested were methyl cellulose, sodium alginate, gelatin and agar. The first two were very difficult to handle and seemed slightly toxic for the leptospires, but gelatin and agar seemed quite suitable.

The action of antiserum on leptospires immobilized by gelatin or agar.

The usual series of dilutions of the antiserum were made in tubes using 25 p.c. gelatin in Vervoort's base (adjusted to pH 7·5) as the diluent, so that the final concentration of gelatin in the antiserum-spirochaete mixture was about 11 p.c. One set of dilutions was solidified in ice-water at once and then incubated at 17° C., the other was incubated at 30° C., at which temperature the gelatin was, of course, still fluid. Two control series of dilutions, without gelatin, were also incubated at these two temperatures.

At 30° C. the only effect of the gelatin was to slow down the transformation of agglutinated leptospires into "lysis balls". In view of the increase in viscosity of the medium due to the gelatin, it is only to be expected that the reaction would be slower. The leptospires in the tubes kept at 17° C., even after incubation overnight, showed nothing but the preliminary stages of agglutination when a tube was melted in water at 37° C. and a drop quickly examined under the darkground microscope. Clumping of the now freely-moving leptospires could be followed readily, but no evidence of lysis or of degeneration was to be seen even under the oil-immersion objective.

Slide preparations were made by mixing a drop of a well-grown culture with a drop of antiserum diluted in 25 p.c. gelatin in Vervoort's base. The dilution of antiserum chosen was one that gave good development of "lysis balls" in the usual tube test. The drops were immediately sealed under a coverslip, the slide put on ice so as to set the gelatin quickly, and then incubated at 17° C. The individual leptospires were fairly well fixed in position by this means. Similar preparations were incubated at 30° C. as a control, as well as controls without gelatin which were incubated at 17° C. All the controls showed numerous "lysis granules" with leptospires projecting from them, but no lysis, or agglutination (save of a very minor nature), was to be seen in the gelatin preparations incubated at 17° C.

The leptospires could also be successfully immobilized with agar. A 2 p.c. solution of Bacto-agar or a 1 p.c. solution of New Zealand agar in Vervoort's base was melted, cooled to 45° C., and used as the diluent for the antiserum. Drops of these agar-serum dilutions were then mixed with drops of a suspension of leptospires on slides, flattened under a coverslip and cooled quickly so as to

set the agar. Apparently the leptospires were not killed during their brief exposure to the warm agar as many of them could be seen still twisting and writhing, although their translational movement was very small in this concentration of agar. The slide preparations were incubated at 30° C. and at room temperature, but no evidence of lysis was seen in any of them. The leptospires, even under sealed coverslips, survived quite well for 5–6 hours, but were usually motionless after overnight incubation, as indeed was the case with the controls without added antiserum or without agar.

There is a remote possibility that agar (and gelatin) may inhibit the lysis of the leptospires. As against this it was found that leptospires were still readily lysed by sodium tauroglycocholate (B.D.H.) in the presence of agar.

As lysis is supposed to take place only at certain concentrations of the antiserum, it was decided to try the effect of allowing the antiserum to diffuse into a column of agar in which the leptospires were immobilized. Antibodies are known to diffuse quite readily through agar so that in some zone, at least, the antibody should reach a concentration suitable for the lysis of leptospires. A drop of a culture of leptospires, concentrated by centrifuging, was mixed with a drop of 1 p.c. of New Zealand agar in Vervoort's base on a sterile slide and covered with a 1″×½″ sterile coverslip which was sealed with sterile vaseline on three sides, leaving one of the ½-in. sides unsealed. The slide was placed in a screw-cap bottle so that the lower unsealed edge of the coverslip was immersed in the serum, a 1/10 or 1/100 dilution of rabbit antiserum (titre 1/10,000), or a 1/10 dilution of normal rabbit serum to act as a control. No lysis was observed after incubating the slides for 5 hours at 30° C., or even after 3 days at 20° C. The number of leptospires and their appearance seemed to be the same regardless of their distance from the edge of the coverslip closest to the antiserum and also showed no differences from the controls. In order to check these results a similar diffusion experiment was set up using tubes instead of slides. The tubes, of approximately 6 mm. diameter, were coated with 0·5 p.c. New Zealand agar as uniformly as possible and allowed to dry. They were then filled with a mixture of equal volumes of leptospiral suspension and 1 p.c. New Zealand agar in Vervoort's base to a height of 2–3 cm. and then immediately set in cold water. Antiserum (titre 1/10,000) diluted 1/10 was then layered on top of the column of agar to a depth of 1 cm. After the tubes had been incubated at room temperature for 48 hours the serum was pipetted off, the top of the column washed with Vervoort's base, and the tubes immersed in 10 p.c. formalin. The column of agar was found to slide out very readily and was then sliced into discs approximately 1·25 mm. thick by means of a razor and a metal guide which contained a hole just large enough to allow the column of agar to be inserted into it. These agar discs were only approximately the same volume for the agar was very easily compressed while being cut and the degree of compression varied. Samples were taken from the top 0·5 cm. and from about the

1 cm. level. Each agar disc was melted after the addition of 0·1 ml. water and the number of leptospires were counted in a Petroff Hausser counting chamber under darkground illumination. The counts were rather variable, as may be expected in view of the differing sizes of the sample discs of agar as well as the normal error of counting, but they were of the same order; e.g., in one experiment with a mean of 359 the range was 307 to 423. They did not show the very large differences to be expected if lysis had taken place.

The conclusion reached from these experiments was that immune serum does not lyse individual leptospires. Perhaps lysis can only take place after the leptospires have been clumped together, although this seems unlikely. The most plausible explanation of the apparent lysis seems to be that leptospires have not disappeared but are still present in the densely compacted "lysis balls."

The effect of complement on the agglutination-lysis reaction.

Complement must be present if an antiserum is to exhibit its bacteriolytic (or bacteriocidal) powers, so, although Schnüffner and Mochtar (1927) had found that it was not necessary for the lysis of leptospires, it seemed worthwhile to re-examine its effect on the spirochaete-antiserum system in view of the failure of the previous experiments to demonstrate unequivocal lysis.

An agglutination test was set up in the usual way save that guinea-pig complement (titre 1/110:50 p.c. haemolysis endpoint) was added to each tube so as to give a final dilution of 1/10 and that the antiserum, an antipomona rabbit serum, had been inactivated at 56° C. for 30 minutes. The results were read after 4 hours at 30° C. and again after overnight incubation at 4° C. A control was set up exactly as above save that the guinea-pig complement was inactivated by heating. The titres of both test and control were the same (1/10,000) and the only difference noted was that, in the presence of complement, the leptospires clumped into loose floccules up to the 1/100 dilution as against only 1/10 in the control. In the higher dilutions the usual "lysis balls" were seen. The experiment was repeated using the serum from a cow naturally infected with *L. mitis* as the antibody and a culture of the same serotype as antigen. The results obtained were the same whether complement was present or not.

As a check on these results suitably diluted immune serum, guinea-pig complement and leptospiral suspension were mixed on a slide and sealed under a coverslip. In some of the experiments the leptospires were immobilized with gelatin or agar. Controls in which the complement had been inactivated were also set up. All slides were incubated at 30° C. or at 17° C. for periods of up to 18 hours. No evidence of lysis could be seen although, of course, when the leptospires were not immobilized by agar or gelatin, "lysis granules" were formed. Certainly no difference could be distinguished between the preparations with added complement and those without it.

The formation of "lysis balls" by immune serum.

Although no evidence has been found that leptospires are ever really lysed, it cannot be denied that there is a marked difference between the loose network usually formed by the agglutinating leptospires in the lower serum dilutions and the closely packed clumps that have been misnamed "lysis balls". The concentration of serum protein is of course greater in these lower dilutions and the possibility that this was the cause of the different behaviour of the leptospires was explored by setting up tests in which the amount of serum was kept constant by the addition of the appropriate amount of normal rabbit serum to each dilution. However, the results were no different from usual.

Another possibility is that floccule formation takes place when antibody is in excess, and "lysis ball" formation when antigen is in excess. To test this an agglutination test was set up in centrifuge tubes and, after incubation overnight at room temperature, the leptospires were spun down in an angle centrifuge at 6,000 *g.* for 30 minutes. The supernatants were then tested for residual antibody, and this was found to be still present in most of the dilutions which had shown "lysis ball" formation as well as in those where loose floccules had been seen.

Formolized suspensions of leptospires never form "lysis balls", only loose floccular clumps right out to the titre of the antiserum. Although it seemed likely that this was a general phenomenon, namely, that dead leptospires never form "lysis balls", it was decided to test the effect of immune serum on leptospires killed in a number of different ways. Accordingly suspensions of leptospires killed by the addition of 0·0005 p.c. methylene blue, 0·25 p.c. phenol, 0·01 p.c. mercuric chloride, and by heating at 56° C. for 45 minutes were put up against the usual dilutions of antiserum in parallel with a suspension of living leptospires and one killed with 0·5 p.c. formalin. As expected, all the killed suspensions gave similar results: no "lysis balls" were formed, only loose clumps of leptospires.

The action of formalin on the leptospires is very quick and advantage was taken of this fact to see what what would happen if the leptospires were killed at various times after the beginning of their reaction with immune serum. Under these conditions Rimpau (1943) claimed that lysis was invariably hindered if the formalin was added after 2½ to 5 minutes, although not always if its addition was delayed for 10 or 15 minutes. After 20 minutes the addition of formalin had no effect. The final results were read after 24 hours. Rimpau believed that the fixation of lysins began after 5 minutes and was complete after 20 minutes.

An antiserum (titre 1/10,000) diluted 1/500 was mixed with an equal volume of the leptospiral suspension in each of seven test-tubes and incubated at 25° C. Neutralized formalin to give a final concentration of 0·5 p.c. was added after 0, 5, 10, 20, 40 and 60 minutes respectively. A drop from each

tube was examined under the darkground microscope after incubation for 0, 2½, 5, 10, 20, 40 and 60 minutes, and finally after 4 hours.

It was found that the formation of "lysis balls" promptly came to a stop as soon as formalin was added. The balls that had already been formed maintained their usual structure, a dazzling-white central mass with or without an attendant wreath of leptospires, and were then slowly incorporated into the large loose clumps typically found when formolized suspensions are used, i.e., loose floccules were present which incorporated refractile granules or balls. The living leptospires reacted much more quickly than the formolized, which is to be expected since the living leptospires are in active movement and so collisions are more frequent. In the non-formolized control after 2½ minutes approximately half the leptospires were free in the field (++), after 10 minutes only a quarter (+++), while after 40 minutes only a few individual "survivors" could be seen (++++). The tube formolized immediately after mixing was negative after 2½ minutes, and had only reached a ++ reaction after 20 minutes and a +++ after 60 minutes. The two tubes that were formolized after 40 and 60 minutes preliminary incubation showed the same picture as the control, confirming the fact that the reaction was virtually complete (at this dilution of this particular antiserum) after 40 minutes. The only change noticed after this time was a tendency for the "lysis balls" to stick together to form rather bizarre clumps. Thus it seems confirmed that "lysis balls" are formed only by living leptospires: as soon as formalin is added, even though the smaller "lysis balls" are incorporated into clumps, no new or large "lysis balls" are formed. Presumably in Rimpau's experiment, lysis was unaffected when formalin was added 20 minutes after the start of the reaction because "lysis ball" formation was virtually complete at this time.

DISCUSSION.

The foregoing experiments failed to demonstrate unequivocal lysis of leptospires by immune serum even when complement was present. Previous workers all seem convinced that lysis in some form or another does take place so some explanation of these conflicting results must be attempted. It is unlikely that any peculiarity of the strains or antisera used was to blame as a number of each was used. A partial explanation may be that the presence of "lysis balls" is easily overlooked when, as is usually the case, serum and antiserum are mixed in test-tubes or in the depressions in an artist's pallette since the bigger "lysis balls" sink to the bottom and may be left behind when the loopful is taken for examination under the microscope. If the whole of the reaction takes place in a drop on the slide as in Krüger's method, the presence of "lysis balls" can hardly be overlooked, although in this method the drops are quite large and extend over several microscope fields under the low power objective. However,

from Krüger's description (Krüger, 1953) it appears that clumping and formation of "lysis balls" always preceded the "lysis" of the leptospires. He mentioned a diminution of the agglutinated leptospires as evidently due to the destruction of the clumps by lysis, but, of course, the picture described could be just as well explained by the leptospires becoming more closely aggregated so that the clumps appeared smaller. Rimpau (1943) considers that lysins are substances quite distinct from agglutinins and indeed claims to have prepared a purely lytic serum by absorbing the agglutinins from a patient's serum with a formolized suspension. On the other hand, an antiserum prepared by the inoculation of a formolized culture contained lysins as well as agglutinins and both could be absorbed from it by a formolized suspension. Rimpau attributes the difference in the antisera to the different immunizing agent, an infection in one case and killed organisms in the other. However, the currently accepted view in immunology is that the same kind of antibody acting upon the antigen under different circumstances causes the different serological effects such as agglutination, precipitation and bacteriolysis. Certainly in my experience, suspensions of formolized leptospires absorbed both the "lysins" and the "agglutinins" from the sera of infected animals (and also from the sera of rabbits artificially immunized by the injection of living leptospires).

There has been some disagreement between different schools of workers about the criterion to adopt for reading the final titre of an antiserum. Bessemans and his co-workers (1938, 1939), while not denying that lysis does take place, claim that agglutination is found in the highest dilutions and that the titre is to be taken as the point where "terminal agglutination" ceases, while the Dutch workers (*cf.* van Thiel, 1948, p. 27) think that this "terminal agglutination" is non-specific and that the clumps are the so-called "brutnester" of Schnüffner which are to be found at times in normal cultures. These differing viewpoints may be reconciled to some extent by assuming some confusion as to what is to be called lysis and what agglutination. Bessemans, who usually read his results by placing a coverslip over the drop on the slide and observing with a 10× eyepiece and a 1/12 in. oil immersion objective, could see the detailed structure of the clumps of leptospires and so probably called agglutination what a Dutch worker would have called lysis. If immune serum does not lyse leptospires much of this disagreement vanishes.

Schnüffner and Mochtar (1927) found that the leptospires that survived "lysis", when transferred to fresh culture medium gave rise to descendants that were just as susceptible to "lysis" as those in the original culture. This is rather surprising if lysis is really taking place; it would be expected that a lysis-resistant (and presumably antigenically different) strain could be selected. However, it is to be expected if these "unlysed" leptospires are only those which by reason of failure to make contact with other leptospires have not been incorporated into "lysis balls."

If then there is no lysis of leptospires, how can the different appearance of the "lysis ball" and the loose floccular clump, as typically found in the lower dilutions and with formolized suspensions be explained? "Lysis balls" are formed only by living leptospires and when a "lysis ball" is torn apart, the leptospires are seen to be alive and in active movement. Perhaps the "lysis ball" is formed by the leptospires, clumped together by the antiserum, writhing and twisting until they are knotted into a compact mass. Indeed this process can be followed under the microscope in mixtures of antiserum and leptospires on a slide. Also, if a formolized suspension is shaken with antiserum in a shaking machine while agglutination is taking place, the clumps are much tighter and denser than usual, so much so that they have a central nucleus that is as refractile and dense looking as a "lysis ball". However, the edge of the clump presents a very ragged appearance even under the low power objective owing to projecting clumps of leptospires in contrast to the more or less smooth-looking edge of the typical "lysis ball."

On this explanation living leptospires would be expected always to form "lysis balls" in the presence of homologous antiserum whereas in the lower dilutions they usually form loose floccules. This type of clumping is not due to the increased concentration of serum protein present, nor is it due to the leptospires being killed by the antiserum, as may be confirmed by direct examination under the microscope. However, they do seem to move less actively in these floccular clumps and so are perhaps sufficiently affected by the more concentrated antiserum to hinder the active twisting and coiling movements necessary for close binding into "lysis balls."

SUMMARY.

When living leptospires were mixed with various dilutions of an antiserum they usually formed loose floccular clumps in the lower dilutions, but in the higher dilutions these floccules were replaced by dense refractile spheres, the so-called "lysis balls."

These two different types of clumps could not be related to antigen or antibody excess or to the varying amount of serum protein present. Leptospires, when dead, formed only the loose floccular clumps, but living leptospires were not necessarily killed by those dilutions of antiserum which caused the formation of this type of clump.

Contrary to the usual belief, leptospires in the "lysis balls" did not appear to be dead or dying and showed no sign of lysis even in the presence of complement. Leptospires prevented from clumping by incorporation in an agar or gelatin medium also showed no evidence of lysis by antiserum.

REFERENCES.

Bessemans, A., Wittebolle, P. and de Borchgrave, O. (1938): Rev. belge Sci. méd., 10, p. 238.
Bessemans, A., Wittebolle, P. and Devuyst, R. (1939): Z. Immun Forsch, 97, p. 309.
Krüger, A. (1953): *Ibid.*, 110, p. 17.
Rimpau, W. (1943): Zbl. Bakt. (1 Abt., Orig.), 149, p. 65.
Schnüffner, W. and Bohlander, H. (1939): *Ibid.*, 144, p. 434.
Schnüffner, W. and Mochtar, A. (1927): *Ibid.*, 101, p. 405.
Thiel, P. H. van (1948): The Leptospiroses. Leiden: University Press.
Uhlehuth, P. and Fromme, W. (1930): Handbuch der pathogenen Mikro-Organismen, 3rd ed., vol. 7, p. 597. Jena: Fischer.
Wolff, J. W. (1925): Arch. Schiffs- u. Tropenhyg., 29, p. 111.

OBSERVATIONS ON TACHYPHYLLAXIS PRODUCED BY PITOCIN INJECTIONS IN THE FOWL

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Injection of oxytocic fractions (pitocin) of posterior lobe pituitary extract into anaesthetized fowls evokes a transient depression of blood pressure. A second injection of the same dose, as soon as the base pressure has returned to its initial level after the first dose, evokes a smaller depression. Further serial doses lead progressively to greater insensitivity. The insensitive state can also be evoked by one massive injection. This phenomenon is referred to as tachyphyllaxis, or, alternatively, the preparation is said to exhibit increasing tolerance to pituitary extract. Strahan and Waring (1954) have reviewed earlier work on pituitary tolerance and recorded new observations.

The following possible explanations of the tolerant state appear to be easily susceptible of test:

(a) A substance which either inhibits relaxation of the vascular muscles, or inactivates subsequently injected pituitary, is released into circulation in response to pituitary injection. The progressive development of the refractory state during close serial injections implies that there would be only slow loss of this substance by destruction, inactivation or excretion.

(b) Receptors on the vascular muscle fibres are inactivated.

(c) An essential substrate is exhausted.

(d) Special areas of the vascular bed remain in prolonged relaxation after injection and the initial base pressure is restored by compensatory vasoconstriction in another special area. Complete tolerance would then result when the first special area attained its morphologically maximum dilatation. An alternative, a variation of the same theme, is to imagine that when vasodilation has been evoked by pitocin, another neurohumoral mechanism is brought into play so that the same blood vessels are returned to their original state of tone. This, of course, would not achieve any explanation of progressive insensitivity unless we also assumed that the new neurohumor overrode the next injection of pitocin.

[1]Working under a grant from the National Health and Medical Research Fund.

None of the above are, theoretically, mutually exclusive.

The experiments to be described were designed to test (a) only. They do, of course, automatically test (b) also and have relevance to (d). The essential desiderata of such an experiment are twofold:

(i) Transfer of blood from a tolerant animal (A) to one not tolerant (B) to show whether a state of tolerance is transferred with the blood.

(ii) Assuming tolerance is transferred from A to B, then it would be necessary to demonstrate that the amount of residual free pituitary in the transfused blood is insufficient to evoke tolerance in B.

The experiments are most readily considered under two headings—preliminary and final.

PRELIMINARY EXPERIMENTS.

Data on the onset and dissipation of tolerance.

Information on the time relations of onset and dissipation of tolerance were pre-requisite for deciding when to transfuse blood so as to have the maximal concentration of putative tolerance factor (T) associated with a non-tolerance inducing level of pitocin (O). Actually, when we came to re-examine previous data, it became evident that not only were time relations unknown, but magnitude of recovery was in doubt.

In the comparable event of tolerance to pressor fraction in mammals, inspection of traces by Jones and Schlapp (1936) shows that after a single big dose, which confers a considerable degree of tolerance, there can be complete recovery of sensitivity with sufficient rest. With birds there is no comparable information for O. Coon (1939) has stated that after a single large dose the preparation is insensitive to small doses for 20 minutes, but the experiments were not continued long enough to show whether complete recovery of initial sensitivity resulted. Similarly, Strahan and Waring (1954), using a close sequence of small doses, demonstrated a considerable recovery of sensitivity with rest, but again did not continue the experiments sufficiently to decide whether complete recovery was possible. It is also apparent from Coon's paper that working with a sequence of moderate doses, a degree of tolerance is attained quickly and this constant level of sensitivity is maintained for some time. Complete description of tolerance would demand an exhaustive sequence of experiments with the following variables in different combinations: (a) size of dose, (b) time intervals between doses, and (c) different base blood pressures; and in all measuring the amount of residual pituitary excitant in circulation.

With regard to our present theme there could be no justification for such a long and costly series. And this is particularly true when we recall that in our definitive experiments check testing of residual pituitary would be undertaken, and that the only way of testing for recovery from tolerance is to inject a small

predetermined test dose, and the injection of this alone must be expected to enhance any tolerance still extant when it is injected. No great precision can therefore be expected in assessing the precise onset of recovery from tolerance. Consequently we chose to perform a limited number of experiments designed to yield specific answers to our most urgent questions. Initially, we used substantially the Coon procedure of dissecting out the major vessels of the leg for cannulation. Later, when we found that experiments had to be continued over 12 hours and it became a reasonable assumption that surgical trauma might be a significant agent in later results, we took elaborate care to seal all minor blood vessels together with the avoidance of cutting through major muscles. Finally, towards the same end, we used wing arteries and veins for cannulation and the cutting was then so superficial that at the end of the experiment the wound was sutured and an uneventful recovery ensued.

The following injection procedure was standardized in all experiments:

(a) 1 or 2 trial doses of 1/4 to 1/6 i.u. were made to ascertain the minimal test dose which would give a good depressor response but which would not be expected to induce tolerance at the intervals selected.

(b) The adopted "test" dose was put in serially at a time interval which experience indicated would not evoke tolerance, usually 10–15 minutes interval, and the experiment was not continued unless it was certain that no tolerance was developing after 6 doses.

(c) Either a large dose or a series of moderate doses in rapid succession were put in to evoke tolerance.

(d) Once tolerant, one injection of test dose was injected immediately to confirm insensitivity and this was repeated at quite arbitrary intervals which experience indicated would give significant information about the prevailing state of tolerance—these trial doses were kept to the minimum so as not to enhance the tolerant state.

Inspection of the traces permits us to draw the following conclusions relevant to the planning of the definitive experiments (*vide infra*):

(i) With small doses widely spaced no loss of sensitivity results. When either the dose is increased or the interval between doses is decreased, sensitivity decreases (i.e., preparation becomes tolerant). This confirms previous findings. Our new experiments clearly show, in addition, that for any moderate dose at a short enough constant interval, sensitivity decreases to a certain level and then does not decrease further. Sensitivity (or tolerance) has then reached a steady state.

(ii) After tolerance has been induced, either by a single large dose or by serial small doses in close succession, there follows a period in which the sensitivity to test doses gradually returns until a constant response is attained. This response is less than the initial responses to the "test" dose. In other words, a plateau has again been reached which lasts for many hours.

(iii) With the ordinary preparation full recovery of sensitivity was never observed [(d) above], but the total time involved in the experiment is such that general trauma and stress may be complicating factors. Consequently, in some experiments we repeated (d) and, then after suturing, let the animal recover. Repetition of the experiment on the same bird 3 days later showed that there is eventually full recovery of initial sensitivity. We have not precisely fixed the time needed for full recovery, but it is more than 24 hours and less than 72 hours.

The plateau reached in (ii) does not appear to be necessarily correlated with the magnitude of the tolerance induced, but may be related to the initial sensitivity of the bird. This latter point is suggested by our traces, but it would need specific experiments of a different design to decide the issue clearly.

On measuring pituitary excitant in bird circulation.

We first attempted to measure the amount of pituitary circulating in the injected bird by withdrawing samples of blood and injecting them into another bird in the same manner analogous to that used by Jones and Schlapp (1936) in their studies on mammalian pressor activity. We were completely unsuccessful. In the terminal stages of the experiment, when circulating pituitary is low, 20 ml. of blood have to be withdrawn from the recipient and injected into the donor. Aside from the fact that withdrawal of such large amounts of blood quickly is inimical to continued good condition in the donor, the direct pressor effect of injecting 20 ml. into the recipient completely masks any small depressor quality the blood is carrying.

We then examined the possibility of using the rat uterus *in vitro* with the saline prescribed by Holton (1948); this was successful. Fig. 1 shows our

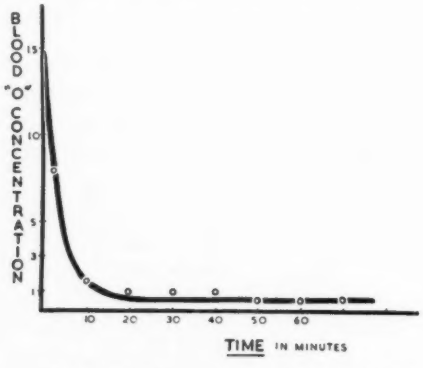


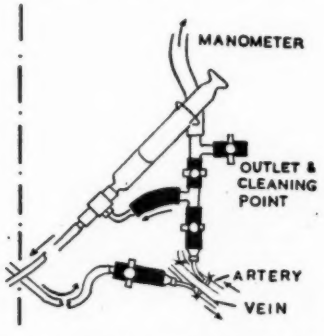
Fig. 1. Blood concentration of pitocin following single large dose (i.e. International units in total blood volume—blood volume taken to be 8 p.c. of body weight—Dukes, 1947).

results. The double ended arterial cannula used in the bird for manometric measurement did not interrupt the flow of blood through the limb and samples for test were withdrawn from the uninterrupted flow passing through the cannula. Early experiments failed because after centrifugation the plasma of uninjected birds was oxytocic. We later found that whole blood allowed to stand for the same length of time, about 10 minutes, showed the same property. However, freshly drawn blood injected immediately into the uterus bath had such low excitant properties that the maximum needed, 5 ml., had no measurable effect in a total bath content of 50 ml. We have not tried to identify the oxytocic excitant noted above; we avoided its complicating effect by immediate assay of blood. Its titre rises with standing; it cannot be histamine which relaxes the rat uterus. Besides the usual precautions taken in an oxytocic assay, we, of course, added the standardizing pituitary doses in the same volume of saline as the volume of blood injected; substituting whole fresh blood as diluent showed no measurable difference from when saline was used.

DEFINITIVE EXPERIMENTS.

Pairs of black Australorp roosters of approx. 3 kilog. were anaesthetized with Na phenobarbitone and their bloods tested for compatibility. They were then dissected, heparinized and their blood streams united with polythene tubing as shown in Fig. 2; all tubing was initially filled with heparinized tyrode solution. The bird to receive doses of O to evoke tolerance is henceforth called A, its partner B. The experimental programme was then:

1. Before any transfusion, serial small fixed doses (1/4–1/6 i.u.) of O, at a



CROSS-CIRCULATION APPARATUS.
FOR ONE BIRD — The second bird is set up as the mirror image of the first.

Fig. 2. Diagram of cross circulation apparatus.

fixed interval insufficient to induce tolerance, were administered to each animal. This dose is the one subsequently used to test for waning tolerance and is henceforth called the "test" dose.

2. Up to 5 mls. of blood were withdrawn from each bird and tested for oxytocic activity in the uterus bath (in no experiment was there measurable activity in this quantity of blood if used immediately).

3. Cross-circulation was effected by two operators pumping 10 mls. of blood at a time from bird A to bird B and *vice versa* for 20 such transfers. The external circuit was then closed at the venous end. The maximum possible transfer (assuming complete mixing) was estimated to be approximately 35 p.c. of the initial blood volume of each bird.

4. A test dose of O was administered to each bird which confirmed that the perfusion *per se* caused no diminution of response.

5. Bird A was made tolerant by the administration of a single 15 unit dose of O.

6. *Immediately* subsequent to this, blood was withdrawn for assay in the uterus bath and this procedure was repeated at 10-minute intervals. Following the initial withdrawal, a test dose was administered to establish that tolerance had been induced.

7. Circulating O in A was determined serially by uterine assay until the value attained was equivalent to approximately twice the test dose dissolved in the complete blood volume of A. (As the test dose is insufficient to evoke tolerance, twice the test dose distributed equally over birds A and B by transfusion cannot evoke tolerance in B, and in fact the recipient received less than half of the donor blood.)

8. A sample of blood was taken from B for uterine assay to confirm that it was still devoid of oxytocic properties. A test dose was administered to B to confirm that it had retained its original sensitivity.

9. Cross circulation was performed as before.

10. A test dose was administered to each animal.

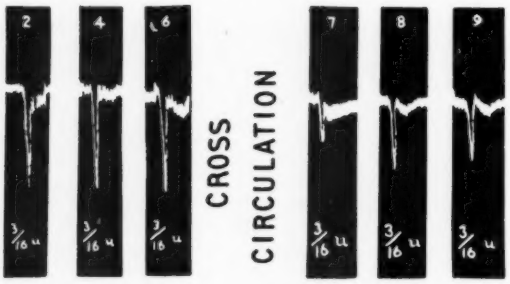


Fig. 3. Responses of recipient bird (B) to test doses before and after cross circulation.

Our traces (Fig. 3) show clearly that when blood in sufficient quantities is exchanged between a tolerant and a non-tolerant bird the latter now exhibits some degree of tolerance to the test dose. This tolerance is in rough accord with the amount of a putative T which could have passed across and sensitivity reappears in the same way as described in the preliminary experiments, that is to a plateau less than the initial sensitivity.

DISCUSSION.

The experiments described show that when a bird is rendered tolerant to O, the blood carries a substance (T) which either renders the vascular muscles insensitive to action by O, or inactivates O itself. The latter is unlikely unless the vascular muscle reaction is quite different from that of uterine muscle because we have also shown that there is no antagonism between tolerant blood and pitocin on the uterine strip *in vitro*. Whether T is produced at the muscle surface or elsewhere is unknown. We have no evidence about the nature of T, but we can speculate that it is unlikely to be an anti-hormone of the usual type because these are considered to be antibodies (see e.g., Thompson, 1944) and oxytocin being now known to be non-proteinaceous and of relative low MW, is unlikely to be antigenic (du Vigneaud, 1953). Consistent with this is Dale's (1909) failure to evoke antibodies by injecting pituitrin into rabbits. Our own experiments showing that, after large doses and rest, further doses have an effect, also point in the same direction.

It is apparent that T persists for many hours in the absence of O. From the results of our preliminary experiments it seems likely that the production of T is roughly proportional to the administered O, although there may be a threshold concentration of the latter below which T production is not initiated. The amount of T liberated by any given dose of O is never sufficient to antagonize *completely* the effect of the same dose of O given subsequently.

Our interpretation of the described observations on tolerance can be shown graphically (Fig. 4).

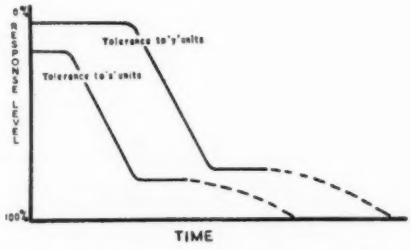


Fig. 4. Interpretation of events during tolerance (see text). The broken lines cover a much greater period of time compared with the solid lines.

Accepting the factual observations and making two likely postulates: that T is initially removed rapidly and, subsequently, there is an exponential loss, and for a given level of tolerance there is a corresponding degree of saturation of the receptors, we can set down the simplest interpretation of the blood concentrations of T, relative to the state of tolerance. Fig. 5 represents these interpretations. On this graph there are superimposed actual responses to pitocin.

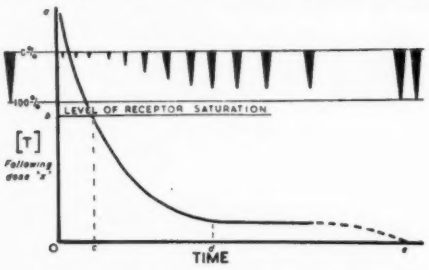


Fig. 5. Putative decline of circulating T following a dose "x" (see text). Dotted line, as in Fig. 4.

This Figure shows the supposed events when the tolerance evoking dose is that of *x* in Fig. 4. When dose *y* is used for evoking tolerance a similar curve applies, but with *a,b,c,d,* and *e* all further from the graph origin O. The implications of this are complex, and with our present information it is fruitless to discuss them fully.

One theoretical explanation of the tolerance factor, compatible with our present experimental evidence, is that the circulating pitocin (O) is "capped" by conjugation (or mildly modified by metabolic activity in the liver for instance) to become T. T attaches to the O receptors but does not induce the O response, and is only slowly released and/or metabolized from the receptor site. A process such as this would account for (i) blockage of the effect of subsequent doses of O, and (ii) prolonged tolerance.

Presumably ordinary mass action laws could operate, accounting to some extent for the variations in tolerance produced by varying dose levels of tolerance inducing O, and for the observation that when a given tolerance exists, the overt response obtained is still determined by the magnitude of the test dose of "O", as it is in a fresh preparation.

There are no doubt many complicating factors affecting the quantitative aspects of tolerance to pitocin, but our observations lead us to believe that the T substance, which we have demonstrated, is of overriding importance in tachyphyllaxis induced by pitocin.

While no direct physiological implication for normal processes in the intact animal would be warranted from such large doses, nevertheless the results imme-

diately open the question whether endogenous pituitary does evoke anti-factors. Such an event would constitute another homeostatic mechanism.

We can now list with certainty the following facts about pitocin action in the fowl: It has a vasodilator action. It initiates the production of a substance (T) either at the same site or elsewhere which is antagonistic to the vasodilator effect. It disappears from the circulation in a considerably shorter time, about 2 hours, than does T and by exponential decay.

The following points are relevant to T: It can circulate freely in the blood and, in addition, must be diffusible. It is developed rapidly. The amount released in response to a given dose of oxytocin will not completely antagonize the same amount of oxytocin subsequently injected. As it is unlikely that it inhibits oxytocin directly, its site of action is probably at the vascular muscle. For a given level of T, there is a corresponding saturation level for the receptors. T is eliminated at a relatively rapid rate, probably following an exponential decay curve although our observations are too incomplete to confirm this. This rate of decay is nevertheless much slower than that of oxytocin. Because in our reciprocal cross circulation experiments the tolerant donor bird does not recover sensitivity any more rapidly as a result of receiving fresh blood, it is unlikely that T antagonism is effected by exhaustion of a blood borne substrate (considered in (c) of the introduction).

SUMMARY.

The time and dose relations relative to onset and decay of pitocin tachyphyllaxis are analyzed.

Cross circulation experiments show that when pitocin is injected a substance (T) is released into circulation which inhibits subsequent response to pitocin injections. The nature and origin of T are unknown, but some of its properties can be deduced.

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REFERENCES.

Coon, J. M. (1939): Archiv. Internat. de Pharmacodyn. et de Therap., 62, p. 79.

Dale, H. H. (1909): Biochem. J., 4, p. 426.

Dukes, H. H. (1947): The Physiology of Domestic Animals, Comstock Pub. Co. Ithaca, New York, 1947.

Holton, P. (1948): Brit. J. Pharmacol., 3, p. 328.

Jones and Schlapp, W. (1936): J. Physiol., 87, p. 144.

Strahan, R. and Waring, H. (1954): Austral. J. exp. Biol., 32, p. 193.

Thompson, K. W. (1944): In "Chemistry and Physiology of Hormones," Edit. Moulton, F.R., Amer. Assoc. Adv. Sci., Washington.

du Vigneaud, V. (1953): J. Amer. Chem. Soc., 75, p. 4879.

SALMONELLA INFECTIONS OF URBAN RATS IN BRISBANE, QUEENSLAND

by P. E. LEE

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(Accepted for publication 3rd August, 1954.)

Rats have been recorded as carriers of Salmonella infections in various parts of the world (for example, Savage and White (1923), England; Verder (1927), U.S.A.; Hatta (1938), Japan; De Assumpcao and Ribas (1943), South America; Mackerras and Mackerras (1949a), Australia; Schmid, Lieb and Velaudapillai (1953), India), and their function as natural reservoirs of these organisms is well known. In association with an outbreak of gastro-enteritis, caused by *S. bovis-morbificans* in Brisbane in 1947-8, Mackerras and Mackerras (1949a) surveyed a series of Brisbane rats, and found an infection rate of 2–2·5 p.c. Smaller surveys have been made since, and recently the opportunity was taken to examine a considerable number of rats, which were being collected for another purpose. The results of all surveys made by the Institute are summarized in the present paper.

Materials and Methods.

The rats were collected by the Brisbane City Council rat control gang and were delivered dead to the Institute, where they were examined immediately on arrival. By sterile dissection of the abdomen, material from both the large and small intestines was obtained, plated directly on SS agar and brom-thymol-blue lactose agar, and inoculated into tetrathionate broth for enrichment. The TT broth was plated after 24 hours on to brilliant-green agar. Subsequent isolation and identification of Salmonella followed lines previously described (Mackerras and Mackerras, 1949b).

The BTB plates were used to search for *Escherichia coli* 0111 (*α coli*) and *E. coli* 055 (*β coli*), which had not been investigated in previous surveys. Four to six lactose-fermenting colonies were inoculated on to 0·1 p.c. glucose-heart infusion agar and incubated at 37° C. for 4 hours, which encourages K antigen formation. Slide agglutination tests were then performed, using 055 and 0111 *E. coli* OB antisera produced in the laboratory. Any positive agglutinations were confirmed by tube agglutination of boiled broth cultures with specific "0" sera.

RESULTS.

In the recent survey, 208 rats were examined between the beginning of January and the end of July, 1953. Of these, 202 were *Rattus norvegicus* and 6 were *Rattus rattus*.

Twelve strains of Salmonella were isolated from 12 rats, 5 being *S. typhi-murium*, 3 *S. oranienburg*, 2 *S. bovis-morbificans* and 2 *S. anatum*. In addition, *E. coli* 055 was isolated twice, this being the first recorded occurrence of these organisms in other than human hosts. No *E. coli* 0111 were found.

The results, together with those of earlier surveys, are shown in the Table.

Pathogens isolated from rats

Year	Number examined	Salmonellas isolated		p.c. Infection
1947-48	241	*S. bovis-morbificans*	1	
		S. typhi-murium	1	
		S. chester	1	
		S. paratyphi C	1	
		S. meleagridis	1	
		S. adelaide	1	
				2·5
1948-49	296	*S. typhi-murium*	2	
		S. paratyphi C	1	
		S. adelaide	1	
				1·5
1949-50	97	*S. bovis-morbificans*	1	
		S. adelaide	1	
				2
1953	208	*S. typhi-murium*	5	
		S. oranienburg	3	
		S. bovis-morbificans	2	
		S. anatum	2	
		In addition:—		
		E. coli 055	2	
				7
Total	842		26	3

DISCUSSION.

The incidence in various surveys varied from 1·5 p.c. to 7 p.c. The higher incidence recorded in 1953 may be real, or may be, at least in part, the expression of improved methods of isolation now available.

It was also found that Salmonellas could be isolated more readily from the large than the small intestine, as shown in the following figures:

From small intestine only	0
From large intestine only	8
From both	4

The rats investigated were collected from all over Brisbane. There was no indication of any special focus of infection in any one locality, nor of any difference between rats from clean suburbs and relatively squalid city areas. This indicates that the high incidence rate was not associated with poor living conditions or over-crowding, and eliminates any false impression that might have been gained by the study of a series of rats from a restricted area, due to the ease

with which infections are transmitted within colonies of these animals (Welch, Ostrolenk and Bartlam, 1941).

These facts, together with the isolation of a pathogenic type of *E. coli*, give fresh emphasis to the potential danger of rats in spreading intestinal infections, and once again emphasize the importance of rigid rat control campaigns, especially in populated areas.

SUMMARY.

In all, 842 rats, collected from various parts of Brisbane, have been examined. Salmonella isolations were made from 24 of these, and represented eight types—*S. typhi-murium, S. chester, S. paratyphi C, S. oranienburg, S. bovis-morbificans, S. meleagridis, S. anatum* and *S. adelaide*. In addition, two isolations of *E. coli* 055 have been made.

Acknowledgments. The writer is indebted to Dr. E. Singer, of this Institute, for the records for 1949-50, to Miss N. Atkinson, University of Adelaide, for identifying the Salmonellas isolated, to Mr. C. Ludford, of this Institute, for confirming the identity of the strains of *E. coli* 055, and to the City Council rat control gang for supplying material for examination.

REFERENCES.

De Assumpcao, L. and Ribas, J. C. (1943): Mem. Inst. Butantan, 17, p. 127.
Ghosal, S. C. (1941): Indian med. Gaz., 76, p. 489.
Hatta, S. (1938): Jap. J. exp. Med., 16, p. 201.
Mackerras, I. M. and Mackerras, M. J. (1949a): J. Hyg., 47, p. 166.
Mackerras, I. M. and Mackerras, M. J. (1949b): Med. J. Austral., 1, p. 1.
Meyer, K. F. and Matsumura, K. (1927): J. infect. Dis., 41, p. 395.
Savage, W. G. and White, P. B. (1923): J. Hyg., 21, p. 258.
Schmid, E. E., Lieb, F. L. and Velaudapillai, T. (1953): Ceylon J. med. Sci., 8, p. 177.
Verder, E. (1927): Amer. J. publ. Hlth., 17, p. 1007.
Welch, H., Ostrolenk, M. and Bartram, M. T. (1941): *Ibid.*, 31, p. 332.

SALMONELLA INFECTIONS OF AUSTRALIAN NATIVE ANIMALS

by P. E. LEE and I. M. MACKERRAS

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(Accepted for publication 3rd August, 1954.)

In March, 1950, an outbreak of enteritis occurred, with several fatalities, in a colony of jew lizards (*Amphibolurus barbatus* Cuv.), which was being maintained at the Institute for parasitological work. The isolation of strains of Salmonella from these, as well as from other Agamid lizards showing no clinical sign of infection, led us to consider the possibility that there might be a reptilian reservoir of Salmonella infections in this country. The work here reported was therefore undertaken, and was extended to include an investigation of such other native animals as became available. All strains isolated were submitted to Miss Nancy Atkinson for identification, and some of the types recorded here have already been listed in papers from her laboratory (Atkinson *et al.*, 1952, 1953).

Materials and Methods.

The animals examined were collected from areas within 60 miles of Brisbane. Some were examined immediately on arrival at the Institute, others after being held in captivity for some time.

Two methods were used to obtain material for culture: (1) Rectal swabbing of live animals. (2) Sampling of intestinal contents obtained by sterile dissection of dead animals and of insects killed by chloroform.

Specimens obtained by these methods were cultured on SS agar, usually on McConkey agar also, and seeded into tetrathionate broth, from which a subculture was made on to brilliant-green lactose agar after 24 hours incubation at 37° C. (Galton and Quan, 1944). Subsequent procedure was on lines indicated in the previous paper (Lee, 1955).

RESULTS.

General survey of vertebrates.

Table 1 gives the results obtained in the survey of vertebrates. Among the mammals, both the *Wallabia dorsalis* (Gray) (black-striped wallaby) from which *S. anatum* was isolated, and the *Isoodon obesulus* (Shaw) (short-nosed bandicoot), which yielded *S. lexington*, died in captivity, the former at the Botanical Gardens Zoo, and the latter at the Institute. These infections were, therefore, probably acquired, as was *S. newport*, which was isolated some time after capture from a bandicoot which was negative on primary examination.

TABLE 1

Salmonella survey of native vertebrates

Hosts		No. examined	Salmonellas isolated	No.	Total
MAMMALIA					
MARSUPIALIA	*Isoodon obesulus*	11	*S. lexington*	1	
			S. newport	1	
			S. norwich	1	
			S. Group E	1	4
	Dasyurus quoll	1	—		—
	Phascolarctos cinereus	1	—		—
	Wallabia dorsalis	1	*S. anatum*	1	1
RODENTIA	*Rattus culmorum*	13	—		—
	Total:	27	5 types		5
REPTILIA					
CHELONIA	*Chelodina longicollis*	2	—		—
	Emydura latisternum	2	—		—
	Chelonia depressa	1	—		—
	Chelone mydas (young)	3	—		—
OPHIDIA					
Pythonidae:	*Python spilotes variegatus*	1	—		—
Colubridae:	*Dendrophis punctulatus*	1	—		—
	Boiga fusca	1	—		—
Elapidae:	*Denisonia* sp.	1	—		—
	Demansia textilis	1	—		—
	Pseudechis porphyriacus	1	—		—
LACERTILIA					
Varanidae:	*Varanus varius*	1	—		—
Geckonidae:	*Peropus variegatus*	2	—		—
Pygopodidae:	*Pygopus lepidopus*	1	—		—
Scincidae:	*Egernia bungana*	2	—		—
	Egernia sp.	1	—		—
	Tiliqua nigrolutea	1	—		—
	Hemispherodon sp.	1	—		—
	Trachysaurus rugosus	1	—		—
Agamidae:	*Chlamydosaurus kingii*	2	*S. rubislaw*	1	
			S. muenchen	1	2
	Physignathus lesueurii	4	*S. chester*	1	
			S. adelaide	1	
			S. rubislaw	1	3
	Amphibolurus barbatus	36	*S. rubislaw*	7	
			S. chester	5	
			S. potsdam	5	
			S. muenchen	4	
			S. adelaide	4	
			S. waycross	2	
			S. bonariensis	1	
			S. birkenhead	1	
			S. derby	1	30
	Total:	66	9 types		35

In addition, 10 frogs (5 *Hyla phyllochroa* and 5 *Mixophyes fasciolatus*) and one eel (*Anguilla australis*) were examined with negative results.

TABLE 2

Salmonella infections in Agamid lizards

No.	Date collected	Date examined	Material	Salmonellas isolated
		Amphibolurus barbatus Cuv.		
3*	? -49	29. 3.50	P.M. Small intestine	—
			Large intestine	—
			Heart blood	—
			Liver	*S. birkenhead*
4*	? -49	27. 3.50	P.M. Large intestine	*S. chester*
5*	31. 1.50	27. 3.50	P.M. Large intestine	*S. chester*
8*	31. 1.50	15. 3.50	P.M. Small intestine	*S. chester*
15	31. 3.50	23. 5.50	Rectal swab	*S. adelaide*
16	31. 3.50	9. 5.50	P.M. Intestine	*S. chester*
			Liver	*S. muenchen*
			Heart	—
21	31. 3.50	23. 5.50	Rectal swab	*S. adelaide*
		10.11.50	P.M. Liver	*S. bonariensis*
			Intestine	—
22	31. 3.50	23. 5.50	Rectal swab	—
		29. 5.50	Rectal swab	—
		19. 6.50	P.M. Heart blood	—
			Liver	—
			Small intestine	—
			Large intestine	*S. adelaide*
33	1. 6.50	1. 6.50	Rectal swab	—
		21.12.50	P.M. Liver	*S. muenchen*
			Large intestine	—
34	31. 3.50	1. 6.50	Rectal swab	*S. chester*
38	15. 8.50	1.10.50	P.M. Large intestine	*S. potsdam*
				S. rubislaw
40	5. 9.50	21.10.53	Rectal swab	*S. potsdam*
				S. adelaide
		31.10.53	P.M. Gall bladder	*S. rubislaw*
42	9. 4.51	29. 8.51	P.M. Intestine	*S. muenchen*
46	20.10.52	7.11.52	Rectal swab	*S. rubislaw*
50	18. 8.53	18. 8.53	Rectal swab	*S. rubislaw*
52	13.10.53	13.10.53	Rectal swab	*S. rubislaw*
54	2. 2.54	2. 2.54	Rectal swab	*S. potsdam*
57	8. 2.54	8. 2.54	Rectal swab	*S. derby*
58	8. 2.54	8. 2.54	Rectal swab	*S. potsdam*
				S. rubislaw
				S. waycross
59	16. 2.54	16. 2.54	Rectal swab	*S. waycross*
60	16. 2.54	16. 2.54	Rectal swab	*S. potsdam*
				S. muenchen
63	5. 4.54	5. 4.54	Rectal swab	*S. rubislaw*
		Physignathus lesueurii Gray		
1	8. 2.50	23. 5.50	Rectal swab	*S. adelaide*
3	? . 6.50	26.10.50	P.M. Intestine	*S. rubislaw*
5	13.10.53	13.10.53	Rectal swab	*S. chester*
		Chlamydosaurus kingii Gray		
1	3.11.52	4.11.52	Rectal swab	*S. rubislaw*
		19. 6.53	P.M. Intestine	*S. muenchen*

P.M. = Post-mortem examination.
* = Lizards involved in the 1950 outbreak of enteritis in the Lizard colony.

The strains of *S. norwich* and *S.* gp. E (not yet identified), however, were obtained from animals immediately after capture, and may serve to implicate *Isoodon obesulus* as a natural reservoir of infection.

All the miscellaneous reptiles, frogs and fish were negative. Only odd specimens were available of the individual species, but the total (35) does form some sort of contrast with the results obtained from the Agamidae. These were so striking that they merit separate consideration.

Infections in Agamid lizards.

Of 42 Agamidae examined, 26 (62 p.c.) were infected with Salmonella. Ten of these, namely, *Amphibolurus barbatus* Nos. 50, 52, 54, 57, 58, 59, 60 and 63, *Physignathus lesueurii* No. 5, and *Chlamydosaurus kingii* No. 1, were infected when captured, while the remainder yielded Salmonella strains on later examination. Details of all infections are shown in Table 2, the serial numbers being those of the Institute's lizard collection.

The multiplicity of types (nine were encountered), and the occurrence of more than one type in several lizards, such as *A. barbatus* Nos. 16, 38, 40, 58 and 60, are striking features of the Table, and the ease with which infections may be acquired and exchanged, as illustrated by *A. barbatus* Nos. 22, 33, 40, and *C. kingii* No. 1, emphasizes the fact that the Agamidae are excellent hosts for this group of organisms.

Examination of invertebrates.

Agamidae are active, fast-moving creatures, which live in open savannah forest country. Mr. David Fleay (personal communication), who has observed them closely in the field, considers that their food consists essentially of dandelion heads and insects. In captivity, they have also been observed to feed avidly on such insects as grasshoppers, cockroaches, meal-worms, and even the green vegetable bug (*Nezara viridula*). Series of insects and a few other invertebrates, collected in areas where Agamidae were known to occur, were therefore examined in the hope that some light might be thrown on the source and mode of infection. The following were cultured, with negative results:

Mollusca (land snails)	2
Myriapoda	4
Scorpionida	7
Insecta—	
Grasshoppers	177
Other Orthoptera	22
Coleoptera	28
Hemiptera	3
Total	243

Fifteen of the Orthoptera were wood cockroaches, *Panesthia laevicollis* Sauss., and the grasshoppers included the following species of Acrididae identified for us by Dr. K. H. L. Key:

Pyrgomorphinae
**Atractomorpha crenaticeps australis* Rehn.

Cyrtacanthacridinae
Bermius sp.
Phaulacridium vittatum (Sjöst.)
**Valanga irregularis* (Walk.)
Austracris proxima (Walk.)
Austracris sp.

Oedipodinae
Gastrimargus musicus (Fabr.)
**Oedaleus australis* Sauss.
Locusta migratoria L.
Heteropternis obscurella (Blanch.)
**Pycnostictus seriatus* Sauss.
Ailopus tamalus (Fabr.)

Acridinae
Acrida conica Fabr.
Froggatina australis (Walk.)

*Abundant species.

The numbers of insects examined are not as large as we would wish, but the incidence of natural infection in the lizards is so high that we would expect their source of infection also to be frequently infected. More insects should certainly be tested; but the possibility that the lizards may also be coprophagous is worth more serious consideration than it has so far been given.

DISCUSSION.

It is not yet possible to discuss the infections in marsupials profitably. Atkinson *et al.* (1953) have recorded two additional types, *S. typhi-murium* and *S. muenchen*, from unidentified kangaroos in South Australia, and it seems likely that marsupials will ultimately be found to present much the same general picture as the higher mammals. Field investigations, rather than the study of animals in captivity, will be necessary in order to determine the true host-parasite relationships.

Salmonellas are generally regarded as being pathogens of mammals and birds, and as having an optimum temperature for growth of about 37° C., so it is surprising to find that they are also associated with poikilothermic animals. Nevertheless, they clearly are, and it is apparent from Table 3 that natural infections with quite a wide range of types occur in Colubridae, Helodermatidae, Iguanidae and Agamidae, and probably in Testudinidae also, for the tortoises, from which Boycott *et al.* (1953) isolated 18 types, were shipped

closely packed under conditions which would encourage any Salmonellas originally present in them to spread among the survivors. Moreover, it cannot be assumed that the Salmonella types marked with an asterisk in the Table were acquired after capture. Some (*S. adelaide* impresses us as likely to be a case in point) may well have been natural infections, which persisted through the period between capture and examination. It is a major defect in our knowledge that we still do not know how long the carrier state may last in reptilian hosts.

It is also evident from Table 3 that some types are found frequently in reptiles, whereas others are of more casual occurrence. The *arizona* Group, which sits uneasily on the boundary between the Salmonellas and the Para-

TABLE 3

Salmonella types reported from reptiles

Host group	Country	Salmonella types	Authors
CHELONIA			
Testudinidae	U.S.A. (ex Galapagos Is.)	*S. newport**	McNeil & Hinshaw (1946)
		*S. sandiego**	
	England (ex Morocco)	18 types*, incl. *S. potsdam* *S. waycross* and *S. manhattan*	Boycott, Taylor & Douglas (1953)
OPHIDIA			
Pythonidae	England (ex W. Africa)	*S. takoradi**	Rewell, Taylor & Douglas (1948)
Colubridae	U.S.A.	*S. newport*	Hinshaw & McNeil (1944, 1945)
		S. typhi-murium	
		S. meleagridis	
		S. rubislaw	
		*S. panama**	
		arizona Group	
Viperidae	U.S.A.	*arizona* Group*	Edwards, Cherry & Bruner (1943)
LACERTILIA			
Helodermatidae	U.S.A.	*arizona* Group	Caldwell & Ryerson (1939)
		*S. montevideo**	McNeil & Hinshaw (1946)
Iguanidae	U.S.A.	*arizona* Group	Caldwell & Ryerson (1939)
		*S. manhattan**	McNeil & Hinshaw (1946)
		S. rubislaw	Hinshaw & McNeil (1947, 1948)
		S. chandans	
Agamidae	Australia	*S. rubislaw*	Present paper
		S. chester	
		S. muenchen	
		S. potsdam	
		*S. adelaide**	
		S. waycross	
		S. derby	
		*S. bonariensis**	
		*S. birkenhead**	

* Isolated from animals in captivity.

colons, was established for parasites of reptiles. *S. waycross*[1] is closely related to it, and has been found in tortoises from Morocco and Agamids in Australia. *S. rubislaw* is also of wide occurrence, having been isolated from one family of snakes and two of lizards; it was the most frequent type in our series. *S. newport*, on the other hand, was the most frequent type in Colubrid snakes in California. The same impression, that *S. rubislaw* and *S. waycross* (as the representative of the *arizona* group) may be primarily parasites of reptiles, whereas most of the others represent adaptability to a wide range of hosts, is given by Table 4, which is limited to Australian host records only. It is curious that *S. typhi-murium*, which has the widest host range in homoeothermic animals, was isolated only twice from Californian snakes and not at all from our reptiles.

TABLE 4

Host records in Australia

Types recorded from Agamidae	Other known hosts in Australia
S. rubislaw	Aboriginal child at Palm Is., N.Q. (Lee, in press).
S. chester	Man, pig, dog, rat, chicken.
S. muenchen	Man, cattle, pig, kangaroo, chicken.
S. potsdam	Man, cattle, sheep.
S. adelaide	Man, rat.
S. waycross	Man (Ferris and Hertzberg, 1945).
S. derby	Man, cattle, sheep, pig, dog, guinea-pig, mouse, duck, chicken.
S. bonariensis	Man, chicken.
S. birkenhead	—

A similar variation is seen in pathogenicity. The *arizona* group contains what appear to be well authenticated natural pathogens of *Heloderma* and Iguanidae in North America. Several other types have been isolated from sick or dead animals in captivity, under circumstances which strongly suggested that the Salmonellas may have caused their illness. The first isolations of *S. chester* and *S. birkenhead* from our lizard colony belong to this category. On the other hand, most infections with almost all types encountered have been clinically completely inapparent, whether the reptiles were living in captivity or freely in their natural environment. This last impresses us as probably the more normal condition for most types, pathogenicity being usually an expression, not so much of virulence of the organisms, as of some serious abnormality in the environment of the hosts.

Each of the three phenomena considered above—variation in frequency of types encountered, variation in host range, and variation in pathogenicity, with the carrier state the most usual one—could have been discussed in similar terms for human babies or any other of the higher vertebrates. It seems to us that

[1]Which should more correctly be known as *S. atherton* Atkinson, 1945.

the host-parasite relationships are broadly similar throughout the whole range of hosts, and that to define the Salmonellas as essentially pathogens of warm-blooded animals is true neither of their behaviour nor of their distribution in nature.

It would lead us too far afield to discuss the extraordinarily interesting problem of the biogeography of the Salmonellas, but there remains the question whether the reptilian hosts may act as reservoirs of infection for man or his domestic animals. Hinshaw and McNeil (1944-47) believed that snakes and lizards were reservoirs of infection for turkeys and chickens, and Boycott *et al.* (1953) felt that pet tortoises might be a danger to their owners, although there was no evidence that transmission had occurred. As regards the Australian Agamidae, there is as yet no evidence relating them to infections of domestic stock, either as donors or receivers, and they would have no contacts with man, except in the open forest country where they normally live. There are only two suggestive instances. The *S. rubislaw* infection in the aboriginal child on Palm Island, noted in Table 3, could well have been acquired directly or indirectly from an Agamid host; there is no inherent improbability in this particular case. Also, Ferris and Hertzberg (1945) described an outbreak of mild enteritis caused by a variant of *S. waycross* in troops training on the Atherton Tableland in North Queensland. Miss Atkinson has informed us that our strains from lizards belong to the same biochemical variant, which was noted by Atkinson *et al.* (1950), and which has not so far been isolated from any other source. Agamidae are common in the type of country which was used for Army installations during the war, so the association may have been real. It seems possible, too, that Agamidae living on catchment areas may occasionally contaminate water supplies. At least they should be considered in Australia, along with man and his stock, when contamination is found to have occurred.

SUMMARY.

A survey of 104 native animals for Salmonellas has yielded a high incidence (62 p.c.) of infection in Agamid lizards, the types found, in order of frequency, being: *S. rubislaw, S. chester, S. muenchen, S. potsdam, S. adelaide, S. waycross, S. derby, S. bonariensis, S. birkenhead.*

In an attempt to find the sources of their infection, 177 grasshoppers and 66 other small invertebrates were examined with negative results.

S. anatum is also recorded from a wallaby (*Wallabia dorsalis*), and *S. newport, S. lexington, S. norwich* and *S.* gp. *E.* (unidentified) from bandicoots (*Isoodon obesulus*).

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cross; to Dr. K. H. L. Key, of the Division of Entomology, C.S.I.R.O., Canberra, for identifying the Acrididae; to Dr. M. J. Mackerras, of this Institute, for help with other identifications; and to Mr. T. Lawton, of Mt. Nebo, and Mr. D. Fleay, of West Burleigh, for collecting many of the animals used in this investigation.

REFERENCES.

Atkinson, N. (1945): In appendix to Ferris and Hertzberg (1945), p. 370.
Atkinson, N., Woodroofe, G. M. and Macbeth, A. M. (1950): Austral. J. exp. Biol., 28, p. 367.
Atkinson, N., Geytenbeek, H. G. and Woodroofe, G. M. (1952): *Ibid.*, 30, p. 177.
Atkinson, N., Carter, M. C., Wollaston, J. M. and Wall, M. (1953): *Ibid.*, 31, p. 465.
Boycott, J. A., Taylor, J. and Douglas, S. H. (1953): J. Path. Bact., 65, p. 401.
Caldwell, M. E. and Ryerson, D. L. (1939): J. infect. Dis., 65, p. 242.
Edwards, P. R., Cherry, W. B. and Bruner, D. W. (1943): *Ibid.*, 73, p. 229.
Ferris, A. A. and Hertzberg, R. (1945): Med. J. Austral., 2, p. 368.
Galton, M. M. and Quan, M. S. (1944): Amer. J. publ. Hlth., 34, p. 1071.
Hinshaw, W. R. and McNeil, E. (1944): Cornell Vet., 34, p. 248.
Hinshaw, W. R. and McNeil, E. (1945): Amer. J. vet. Res., 6, p. 264.
Hinshaw, W. R. and McNeil, E. (1947): J. Bact., 53, p. 715.
Hinshaw, W. R. and McNeil, E. (1948): *Ibid.*, 55, p. 870.
Lee, P. E. (1955): Austral. J. exp. Biol., 33, p. 113.
McNeil, E. and Hinshaw, W. R. (1946): Amer. J. vet. Res., 7, p. 62.
Rewell, R. E., Taylor, J. and Douglas, S. H. (1948): Mthly. Bull. Ministry Hlth. Pub. Hlth. Lab. Serv., 7, p. 266.